Studies of the Regulation of Neutrophil Signalling and Function by Soluble E-Selectin

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Declaration

This thesis and the research described herein is solely my own work. All work presented in this thesis, was, unless otherwise acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference. No part of this work has been, or is submitted for any other degree qualification.

Sarah McMeekin, 2007

Abstract

The selectin family of molecules (L-, P- and E-selectin) mediate the adhesive interactions between leukocytes and endothelial cells and are required for recruitment of leukocytes to inflammatory sites. At sites of inflammation, soluble E-selectin is upregulated by proinflammatory mediators and is released into the bloodstream and underlying tissues. Elevated soluble E-selectin levels act to promote neutrophil β_2 integrin-mediated adhesion by prolonging calcium (Ca^{2+}) mobilization.

In this thesis I have investigated the functional consequences of soluble E-selectin upon neutrophil responses to proinflammatory mediators, the effects of soluble E-selectin on Ca^{2+} signalling, and the signalling events regulating this. Specifically, I have investigated the mechanism by which soluble E-selectin acted to prolong intracellular Ca^{2+} ($[Ca^{2+}]_i$). Soluble E-selectin alone was unable to initiate Ca^{2+} signalling but it allowed store operated calcium entry (SOCE) to occur following stimulation with platelet activating factor (PAF) and I have termed this effect permissive SOCE. This increase in $[Ca^{2+}]_i$ in response to soluble E-selectin and PAF was shown to act through a G-protein coupled receptor (GPCR) coupled to pertussis toxin-insensitive $G_{q/11}$. Using pharmacological approaches to define the ion channels involved it was demonstrated that this effect was mediated by canonical transient receptor potential channels (TRPC) due to its sensitivity to specific inhibition by a store operated channel inhibitor, MRS1845 and a TRPC inhibitor, Gd^{3+}. RT-PCR and western blotting analysis demonstrated that TRPC6 was the principal TRPC family member expressed by human neutrophils. In terms of mechanism, soluble E-selectin activated Src family tyrosine kinases, an effect that was upstream of PI 3-kinase in a signalling pathway that regulates permissive SOCE following exposure of neutrophils to PAF. I have also shown a regulatory role for protein kinase C in control of the soluble E-selectin mediated prolongation of elevated Ca^{2+}.

Soluble E-selectin induced alterations in TRPC channel function acts to promote adhesion, reduce migratory capacity and augment neutrophil destructive potential and neutrophil survival. Soluble E-selectin was demonstrated to bind via the lectin domain, presumably through a putative E-selectin receptor present on neutrophils. Ligation of CD66 with antibodies caused prolonged PAF-induced Ca^{2+} mobilization in a similar manner to that caused by soluble E-selectin. In summary, this thesis describes studies which provide the first evidence for communication between an adhesion receptor and inflammatory mediator at a molecular level. Selectin receptor ligation allows permissive SOCE to occur following PAF stimulation of human neutrophils.

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List of Abbreviations

AM	Acetoxymethyl ester
ARDS	Acute Respiratory Distress Syndrome
DAG	Diacylglycerol
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular Ca^{2+}
cDNA	complementary DNA
CEA	Carcinoembryonic antigen
CGD	Chronic Granulomatous Disease
CIF	Calcium Influx Factor
COPD	Chronic Obstructive Pulmonary Disease
CRAC	Ca^{2+} release-activated Ca^{2+}
CRACM1	CRAC modulator 1
DNA	Deoxyribonucleic acid
dNTP	generic term for the 4 deoxyribonucleotide triphosphates: dATP, dCTP, dGTP, dTTP
DTSSP	3,3′-Dithiobis[sulfosuccinimidylpropionate]
DSS	disuccinimidyl suberate
EGF	Epidermal Growth Factor
EDTA	Ethylenediamine tetraacetic acid
EGTA	Glycol-bis(2-aminoethylether)- N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ESL-1	E-selectin ligand 1
Fak	Focal adhesion kinase
fMLP	N-formylmethionyl-leucyl-phenylalanine
FSGS	Focal & Segmental Glomerulosclerosis
Gd^{3+}	Gadolinium (III) Chloride
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor

GPCR	G-protein-coupled receptor
HBSS	Hank's balanced saline solution
HPV	Hypoxic Pulmonary Vasoconstriction
IgG	Immunoglobulin
ILK	Integrin-linked kinase
IL-1	Interleukin 1
IP_3	Inositol (1,4,5)-trisphosphate
IPAH	Idiopathic pulmonary hypertension
LAD	Leukocyte Adhesion Deficiency
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAPK	Mitogen activated protein kinase
MARCK	Myristoylated Alanine-Rich C-Kinase
MPO	myeloperoxidase
mRNA	messenger RNA
OAG	1-Oleoyl-2-acetyl-sn-glycerol
PAF	Platelet-activating factor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK1	phosphoinositide-dependent protein kinase 1
PFA	Parafomaldehyde
PI 3-kinase	Phosphatidylinositol 3 kinase
PI	Propidium Iodide
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PMA	Phorbol-12-Myristate-13-Acetate

PMN	Polymorphonuclear leukocytes
PSGL-1	P-Selectin Glycoprotein Ligand-1
PRP	Platelet rich plasma
RACK	Receptor for Activated C Kinases
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase PCR
SCID	Severe Combined Immunodeficiency
SLe^x	Sialyl $lewis^x$
SOC	Store Operated Channel
SOCE	Store Operated Calcium Entry
STIM1	Stromal interaction molecule 1
Syk	Spleen tyrosine kinase
TBS	Tris-buffered Saline
TNFα	Tumour necrosis factor-α
TRPC	Canonical Transient Receptor Potential Channels
VOCC	Voltage-operated Ca^{2+} channels

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Chapter 1: Introduction

1.1 The Neutrophil

In vertebrates, recruitment of blood-borne leukocytes represents a key event in the body's defence against invading micro-organisms following injury or infection. The polymorphonuclear leukocytes, which include neutrophils, eosinophils and basophils are critical effector cells in both innate and humoral immunity. In particular, neutrophils are capable of internalisation of micro-organisms via phagocytosis which together with production and release of reactive oxygen radicals, digestive enzymes and antimicrobial peptides allow the elimination of infectious micro-organisms. However, these same processes can cause host tissue injury if they are engaged inappropriately or not tightly controlled.

In view of this potential for neutrophil-mediated tissue destruction, there are multiple levels of control for adhesion processes to ensure that inappropriate recruitment of neutrophils does not occur. Neutrophils have been implicated in the pathogenesis of a number of disease states including respiratory distress syndromes, rheumatoid arthritis and asthma (Malech and Gallin, 1987; Kerstjens and Timens, 2003; Caramori and Papi, 2004). In experimental models of acute inflammation, blockade of neutrophil adhesion is associated with a reduction in tissue damage (Albelda *et al.*, 1994), raising the possibility that perturbation of neutrophil recruitment patterns may alter progression of inflammatory responses, leading to generation of chronic inflammatory conditions, fibrosis and loss of organ function. Although deployment of the neutrophil armoury of degradative enzymes and toxic oxidant species is essential for the elimination of invading pathogens and initiation of tissue repair processes, if neutrophil destructive potential is triggered inappropriately, host tissue damage may occur with pro-inflammatory consequences. Excessive inflammatory cell recruitment or inappropriate cell activation may contribute to the development of chronic inflammatory conditions.

Neutrophils are the most abundant white blood cell type present in the circulation, accounting for 70% of all white blood cells (leukocytes). They are derived from pluripotent stem cells in the bone marrow which undergo proliferation and differentiation under the influence of three major haematopoietic cytokines G-CSF, GM-CSF and IL-3 (Berliner, 1998; Boneberg and Hartung, 2002), allowing them to differentiate and mature into fully functional neutrophils. There appear to be several recognisable morphological stages including myeloblast, primary myeloblast, myelocyte and metamyelocyte stages with subsequent formation of non-segmented (band) neutrophils before fully functional segmented neutrophils acquire their characteristic primary (azurophil), secondary (specific) and tertiary granules. The mature neutrophil is distinguished also by a lobulated chromatin dense nucleus.

Neutrophils are released from the bone marrow into the circulation in the healthy adult, at a rate of 1×10^{11} neutrophils per day (Cannistra and Griffin, 1988). During acute inflammation, release of neutrophils from the bone marrow is thought to increase to more than 1×10^{12} per day (Cannistra and Griffin, 1988). Neutrophils are short lived cells and in the circulation have an estimated half life of only 4-6 hrs. The intravascular pool of human neutrophils is composed of one compartment which is circulating and another that is marginated to the vascular endothelium. Marginated neutrophils are mature neutrophils that leave the circulating bloodstream and are delayed along the walls of microvessels, forming a sequestered or "marginated" pool that can be rapidly mobilized back into the circulation (ATHENS et al., 1961; Hogg, 1987). Marginated pools of neutrophils exist in the liver, spleen and lung, where they can live for 1 to 2 days (Bicknell *et al.*, 1994) before they are cleared by apoptosis. A dynamic equilibrium exists between the circulating pools and marginated pools, allowing exchange with each other (Berkow and Dodson, 1987). Stress, such as physical exercise, catecholamine infusion or injury, increases the circulating leukocyte counts by mobilizing this marginated pool (Muir *et al.*, 1984; Foster *et al.*, 1986; Shephard, 2003).

1.1.1 Neutrophil Characteristics

Neutrophils have a mean diameter ~ 7 μm and are characterised by a multilobed chromatin-dense nucleus and a large number of intracellular granules. Initially neutrophil granules were simply divided into two categories, namely peroxidase positive (azurophil or primary) and peroxidase negative (specific or secondary). However, an additional two further granule subgroups can be defined, namely tertiary/gelatinase granules and secretory vesicles (Borregaard and Cowland, 1997). The importance of neutrophil granules is demonstrated in Chediak-Higashi syndrome in which an apparent inability to mount a proper inflammatory response leads to congenital abnormality in neutrophil granule formation (Davis and Douglas, 1972; Kjeldsen *et al.*, 1998).

The azurophil or primary granules vary in size and have an oval or round morphology (Bainton, 1976; Borregaard, 1997). They contain a wide variety of agents believed to be involved in bacterial killing and are primarily released into the phagolysosomal compartment during phagocytosis. Regulated secretion of granule contents is required to avoid detrimental effects upon host tissue. Amongst the many antimicrobial constituents of azurophil granules, one of the most important for effective bactericidal killing is myeloperoxidase (MPO) (Klebanoff, 1999). The enzymatic activity of MPO is responsible for the conversion of hydrogen peroxide to hypochlorous acid, providing the neutrophil with a highly effective microbial capacity. Azurophil granules also contain antimicrobial peptides called defensins, which form a large proportion of the proteins contained in azurophil granules (Chertov *et al.*, 2000). These small antimicrobial peptides are highly toxic to a wide variety of bacteria, fungi and some viruses. Other important constituents include serine proteases such as elastase and cathepsin G, which are involved in hydrolytic degradation of many substrates including bacterial cell walls (Chertov *et al.*, 2000). The second category of granules is specific granules, which are round, oval or elongated in shape and are slightly larger than gelatinase granules. In contrast to azurophil granules, it is thought that specific granule contents can be released extracellularly. They characteristically contain the iron sequestering protein lactoferrin,

important for preventing the growth of ingested bacteria by depriving them of this vital growth nutrient (Spitznagel *et al.*, 1974; Ellison, III, 1994). Specific granules are also an intracellular source of receptors for the small complement fragment iC3b (Sengelov *et al.*, 1994b), laminin (Singer *et al.*, 1989) and N-formylmethionyl-leucyl-phenylalanine (fMLP) (Sengelov *et al.*, 1994a). It is not clear if these receptors are fully functional when they become translocated to the plasma membrane following degranulation. A further important constituent of specific granules is cytochrome b558, a component of the NADPH oxidase, responsible for the generation of superoxide anion (Calafat *et al.*, 1993).

Gelatinase (or tertiary) granules are smaller than specific granules and are principally composed of the protease gelantinase which may be involved in digestion of the vascular basement membrane, allowing the extravasation of neutrophils (Dewald *et al.*, 1982). Similarly to specific granules, gelatinase granules also contain cytochrome b558 and several acid hydrolases but do not contain lactoferrin.

1.2 Neutrophil Receptors

The interaction of the neutrophil with the local micro-environment occurs through the cell surface receptors that are expressed at the plasma membrane. During the recruitment process and before reaching sites of inflammation the neutrophil alters its cell surface receptor repertoire, increase their metabolic rate and acquire a state of alertness referred to as 'priming'. Many of the key molecular mediators of adhesion, migration and phagocytosis are maintained within intracellular granule compartments, in the azurophil and specific granules. In response to exposure to a variety of stimuli derived either from pathogens (e.g. fMLP) or from the host (a range of cytokines and growth factors), the neutrophil is able to rapidly mobilise these granule contents to the plasma membrane. Coupled with this, the neutrophil can control the levels of surface receptor expression through the rapid and specific proteolytic 'shedding' of the ecto-domain of specific receptors through the action of metalloproteases. Together these

processes combine to generate a very different receptor profile that is optimal for destructive capacity.

The functional capacity of neutrophils, including adherence, reorganization of microbial pathogens and response to inflammatory stimuli, migration, phagocytosis and generation of cytokines are mediated by a large variety of surface receptors. Many of these receptors are involved in neutrophil activation, including chemokine receptors (such as e.g. IL-8), immunoglobulin Fc receptors (FcRs), complement receptors (CRs) and multiple receptors for adhesion molecules (selectins, integrins), as well as receptors for bacteria and microbial products, such as Toll-like receptors, lipopolysaccharide (LPS) and fMLP. Through these receptors, neutrophils can be activated and they migrate from the circulation into the local tissue. Expression of these receptors is essential for neutrophil functions such as chemotaxis, phagocytosis, reactive oxygen species (ROS) production and release of cytokines, chemokines and growth factors that potentiate innate immune and inflammatory responses. In general, these receptors can be divided into four main groups according to their biological function: adhesion receptors; chemotactic receptors; phagocytic receptors and cytokine receptors.

1.2.1 Adhesion Receptors

Integrins are transmembrane cell surface proteins that bind intracellularly to cytoskeletal proteins and communicate extracellular signals (Berton and Lowell, 1999). Each integrin consists of a noncovalently linked, heterodimeric α and β chains. The β_2 subfamily of integrins is composed of four family members: CD11a/CD18 (lymphocyte function-associated antigen-1, LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 and CD11d/CD18, which are heterodimers composed of a unique α (CD11) subunit complexed to a common β_2 (CD18) subunit. These integrins have crucial roles in various neutrophil functions that are required for the containment and elimination of infection. They are present in an inactive state on circulating leukocytes, however, following stimulation of neutrophils with various cytokines, a G-protein-coupled

receptor (GPCR) agonist and high valency ligands, they undergo a rapid conformational change that results in their activation, which is required for optimal integrin function. Active β_2 integrins mediate leukocyte adhesion and transmigration across the endothelium, through interactions with ICAM-1 on the activated endothelium. The β_2 integrins, Mac-1 and CD11c/CD18 also promote the phagocytosis of microbes into phagosomes and recognise many pathogens directly (Ross, 2000). The biological importance of β_2 integrins is highlighted in patients that have mutations in the β_2 subunit (Roos and Law, 2001). These patients, referred to as leukocyte adhesion deficiency I (LADI) patients, exhibit increased susceptibility to infections and peripheral blood neutrophilia (Wehrle-Haller and Imhof, 2003). Several studies showed that neutrophils in LAD patients roll normally but do not transmigrate as efficiently through tissues to sites of inflammation and fail to adhere firmly and spread poorly *in vitro* ((Harris *et al.*, 2001; Bunting *et al.*, 2002). LAD patients suffer from recurrent infections of soft tissues and often die before they are more than a few years old.

Human neutrophils were once thought to express only β_2 integrins. However, in 1990 evidence emerged that demonstrated human neutrophils possess both β_1 and β_2 classes of integrins and that both mediate polymorphonuclear leukocytes (PMN) adherence (Bohnsack *et al.*, 1990). Specifically the integrin $\alpha_4\beta_1$ has been shown to be involved in mediating firm adhesion and transendothelial migration (Kubes *et al.*, 1995). The β_1 integrins share CD29 as their common β subunit (Hemler, 1990). This widely distributed family of integrins contains a series of cellular receptors for extracellular matrix proteins including fibronectin, collagen, laminin and vitronectin. $\alpha4\beta1$ (VLA-4, CD49d/CD29) has also been shown to be involved in lymphocyte, monocyte, eosinophil, basophil and natural killer cell adhesion to cytokine-activated endothelial cells (Morris and Ley, 2004; Zhang *et al.*, 2004).

1.2.2. Immunoglobulin Superfamily Receptors

The structural unit of the immunoglobulin superfamily members is the Ig domain. Ig superfamily members that have been identified in neutrophil recruitment include: CD31 (PECAM1), ICAM-1 (CD54) and ICAM-3 (CD50). CD31 is organized into an extracellular amino-terminal domain containing 6 immunoglobulin (Ig)-like repeats, a short hydrophobic transmembrane domain, and a long cytoplasmic tail (Newman *et al.*, 1990). Studies *in vivo* and *in vitro* have demonstrated a role for CD31 in neutrophil transendothelial migration (Muller, 1995). Experiments using mAbs and recombinant CD31 proteins can inhibit neutrophil migration through blood vessels by blocking at the stage of interaction with the basement membrane (Wakelin *et al.*, 1996). CD31 facilitates the diapedesis of leukocytes both *in vitro* and *in vivo* (Muller *et al.*, 1993; Vaporciyan *et al.*, 1993; Bogen *et al.*, 1994), acts as a trigger for up-regulating integrins on leukocytes (Tanaka *et al.*, 1992; Piali *et al.*, 1993; Leavesley *et al.*, 1994; Berman and Muller, 1995), and thus appears to play a role in cell-cell interactions during an inflammatory response.

ICAM-1, a cell-surface protein with five immunogloblulin-like domains, plays an important role in transendothelial migration of leukocytes (Smith *et al.*, 1988; Yang *et al.*, 2005) through its expression on vascular endothelium and binding to β_2 integrins (Marlin *et al.*, 1990). Immunoglobulin domains 1 and 2 of ICAM-1 are involved in binding to LFA-1 (Staunton *et al.*, 1990; Casasnovas *et al.*, 1999), while immunoglobulin domain 3 mediates binding to Mac-1 (Diamond *et al.*, 1991). ICAM-1 is upregulated through synthesis of protein *in vivo* or *in vitro* in response to inflammatory cytokines (Rothlein *et al.*, 1988), phorbol esters (Rothlein and Springer, 1986) or lipopolysacharides (Jersmann *et al.*, 2001). Integrin binding to ICAM-1 is particularly important for firm attachment and migration across the endothelial surface; for example, migration of human neutrophils through a monolayer of umbilical vein endothelium was inhibited >85% by anti-ICAM-1 blocking monoclonal antibodies (mAbs) (Smith *et al.*, 1988). ICAM–3 is unique in the ICAM adhesion protein family in

that ICAM-3 is expressed only on leukocytes and also regulates neutrophil adhesion by binding to LFA-1 and Mac-1. Both ICAM-1 and -3 mediate binding to the β_2 integrins, LFA-1 and Mac-1, and expression of these receptors is of particular importance in regulating neutrophil behaviour in inflammatory responses.

Fc receptors have been identified as a family of receptors that bind to the Fc portion of immunoglobulin molecules and have been proven to be essential for phagocytosis. The interaction of antibody-antigen complexes with cells of the immune system results in a wide range of responses, ranging from effector functions such as antibody-dependent cytotoxicity and phagocytosis to immunomodulatory signals such as regulating lymphocyte proliferation. All these interactions are initiated through the binding of the Fc domain of antibodies of immune complexes to Fc receptors. FcRs exist for every antibody class: FcγR bind IgG, FcαR bind IgA, FcεR bind IgE, FcμR bind IgM and FcδR bind IgD (Ravetch and Bolland, 2001).

Human neutrophils constitutively express two distinct FcγRs: FcγRIIa (CD32) and FcγRIIIb (CD16). FcγRs are critical participants in inflammation and in the immune response by providing an important link between the humoral and cellular immune systems. FcγRIIa is a transmembrane receptor that can initiate many neutrophil inflammatory responses including degranulation and the generation of reactive oxygen intermediates. FcγRIIIb is a glycosylphosphatidylinositol (GPI)-linked protein that can also initiate a number of neutrophil inflammatory responses including antibody-dependent cellular cytotoxicity. FcαRI (CD89) an IgA receptor, is also expressed on neutrophils and FcαRI activation has been shown to initiate several neutrophil responses including phagocytosis, cytotoxicity, respiratory burst, cytokine release and antigen presentation (Morton *et al.*, 1996; Hellwig *et al.*, 2001; Shen *et al.*, 2001).

1.2.3 Lectin-Like Receptors

L-selectin (CD62L), the lymphocyte homing receptor, and CD44, the hyaluronan receptor, are two of the major adhesion molecules expressed by leukocytes of both lymphoid and myeloid origin (Yong and Khwaja, 1990). L-selectin (CD62L) mediates the initial tethering and rolling of leukocytes on endothelial surfaces, which is a prerequisite for leukocyte extravasation from the blood into tissues and also contributes to recruitment of leukocytes from the blood to areas of inflammation (Tedder *et al.*, 1995). PSGL-1 (CD162) is the major P-selectin ligand on neutrophils. This interaction mediates the tethering and rolling of these cells on endothelial cells under physiological flow, an important initial step in leukocyte extravasation (McEver *et al.*, 1995). Interactions between PSGL-1 and L-selectin can mediate neutrophil-neutrophil interactions, which may amplify neutrophil extravasation (Walcheck *et al.*, 1996). CD44 is a transmembrane glycoprotein which contributes to the adhesion of neutrophils to endothelial cells (Lesley *et al.*, 1993). The principal ligand for CD44 is hyaluronan (HA), an extracellular matrix glycosaminoglycan (Lesley *et al.*, 1993). The interaction between cell-surface CD44 and HA has been implicated in leukocyte extravasation at inflammatory sites (DeGrendele *et al.*, 1997). CD44 on the surface of activated leukocytes can function as an adhesion receptor that initiates the attachment of these cells to HA on microvascular endothelium (DeGrendele *et al.*, 1996). In a fashion similar to the interaction between selectins and their carbohydrate ligands, binding of leukocyte CD44 to vascular HA tethers leukocytes to the vessel wall and supports their rolling adhesion (DeGrendele *et al.*, 1996).

1.2.4 Chemoattractant/7TM Receptors

Neutrophils are attracted to inflammatory sites and/or sites of infections through the production at these sites of chemoattractant mediators, for example the bacterial peptide fMLP, leukotriene B_4 (LTB_4), C5a, PAF. Many of these chemoattractants bind to a heptahelical G-protein coupled cell surface receptor on neutrophils. Human neutrophils

express fMLP receptors (FPRs), C5a-, LTB_4- and PAF receptors. As a result of chemoattractant receptor activation, neutrophils are stimulated to move, adhere and de-adhere, rearrange their cytoskeleton and ultimately to phagocytose infectious micororganisms, secrete granules containing degradative enzymes and antimicrobial agents (Haribabu *et al.*, 2000). The signalling mechanisms and responses used by chemoattractant receptors are thus of major importance in determining how neutrophils will respond to any given biologic situation. For example, N-formyl peptides interact with the fMLP receptor to induce neutrophil chemotaxis, phagocytosis, production of superoxide radicals and release of proteolytic enzymes from intracellular granules (Bae *et al.*, 2003). The PAFR and LTB_4R are known to be involved in regulating adhesion to endothelial cells and chemotactic responses. C5a is a strong chemoattractant and is involved in the recruitment of inflammatory cells such as neutrophils, eosinophils, monocytes, and T lymphocytes, in activation of phagocytic cells and release of granule-based enzymes and generation of oxidants, all of which may contribute to innate immune functions or tissue damage (Guo and Ward, 2005). The receptor for C5a plays a critical role in numerous inflammatory conditions (Guo and Ward, 2005). Another characteristic that all these chemoattractant receptors have in common is their ability to induce rises in intracellular calcium ($[Ca^{2+}]_i$) in neutrophils (Fruman *et al.*, 1991). The binding of fMLP, C5a, LTB_4 and PAF to the appropriate receptor activates phospholipase C (PLC), via a G-protein. The resulting production of diacylglycerol (DAG) and phosphoinositides induces the activation of protein kinase C (PKC) and mobilization of $[Ca^{2+}]_i$, which affects several neutrophil functions.

Chemoattractant receptors are G protein coupled-receptors that span the membrane seven times. Ligand binding to the GPCR is coupled to exchange of GDP for GTP bound to the associated G protein heterotrimer and results in the activation by the G protein α and $\beta\gamma$ subunits of signalling effectors. The $G\alpha$ subunits of the αi class are ADP-ribosylated and irreversibly inactivated by pertussis toxin. In neutrophils PAF, fMLP, C5a and LTB_4 work through distinct G protein coupled receptors, PAF and C5a

signal via a pertussis toxin-insensitive G-protein pathways (G_q) and fMLP and LTB_4 through pertussis toxin-sensitive pathways (G_i).

1.3 Neutrophil Recruitment at Sites of Inflammation

Recruitment of neutrophils to sites of tissue injury or infection is the hallmark of the acute inflammatory response. Efficient neutrophil extravasation at sites of inflammation requires a coordinated cascade of adhesive and signalling events (Springer, 1995; Smith, 2000). Molecular specificity in the targeting of neutrophils to sites of inflammation is mediated by selectins, integrins and the immunoglobulin gene superfamily. Based on *in vitro* and *in vivo* observations, neutrophil recruitment may be described as a sequential process having at least three distinct adhesive events (tethering, rolling and firm adhesion), as shown in Figure 1.1. Neutrophils are captured from the bloodstream by tethering via constitutively expressed L-selectin that recognises glycoprotein ligands on adjacent neutrophils and those upregulated in cytokine activated endothelium (Abbassi *et al.*, 1993). Neutrophil rolling is supported by E-selectin and P-selectin that are upregulated on the endothelial plasma membrane in response to cytokine or thrombin and histamine stimulation, respectively (Lawrence and Springer, 1993; Jones *et al.*, 1993). The carbohydrate and epitope specificity of selectin recognition has been determined by blocking adhesion with mAbs to each selectin, recombinant soluble selectins and fucosylated polysaccharides (Kansas, 1996). These interventions are effective at blocking tethering and rolling *in vitro* and they can inhibit the accumulation of neutrophils at sites of inflammation in animal models of acute inflammation (Kansas, 1996). The P-selectin glycoprotein ligand-1 (PSGL-1) is a dimeric mucin-like glycoprotein found on neutrophil surfaces has also been proven to promote cell adhesion by binding to P- and L-selectin (Yang *et al.*, 1999).

The requirement for selectins in primary cell capture and rolling has also been confirmed in transgenic mice deficient in L-selectin, E-selectin, P-selectin and PSGL-1 (Mayadas *et al.*, 1993; Kunkel and Ley, 1996; Xia *et al.*, 2002). It is clear from this literature that

selectins provide for a redundant molecular recognition system that supports both capture and rolling of neutrophils in blood vessels in which the shear rate is high (>100 s^{-1}) and the collisional contact duration with the endothelium is brief (<25ms) (Simon and Goldsmith, 2002). Rolling along the endothelium is thought to allow sufficient time and elicit the activation of neutrophil integrins that then bind to their counter receptors, resulting in firm adhesion (Simon *et al.*, 1998; Chen and Springer, 1999). The selectins will be discussed in greater details in section 1.6.

A critical step in the transition from neutrophil rolling to arrest is the development of firm adhesion, which is predominantly mediated by the activation of membrane expressed β_1 and β_2-integrins. Their participation is required for stable shear resistant and prolonged adhesion (Hynes, 1992; Taylor *et al.*, 1996). Following activation by chemoattractant agents, such as IL-8 or PAF, the neutrophil upregulates adhesion receptors such as CD11b/CD18. Consequently, firm adhesion to the endothelium is secured via binding through LFA-1 or Mac-1 with endothelial cell ligands such as ICAM-1 (Ley, 1992; Carlos and Harlan, 1994; Springer, 1994; Wang and Doerschuk, 2002).

Completion of the process of neutrophil recruitment follows the development of strong interactions between integrins and Ig-superfamily members. Firmly adherent neutrophils are able to migrate over the luminal surface of endothelium by reversible adhesion, which involves the cycling of integrin receptor avidity (Dustin and Springer, 1989). For example, transmigration of neutrophils across the endothelial barrier involves an interaction between leukocyte integrins and endothelial ICAM-3 and CD31 ligation which is associated with β_2 integrins (Berman and Muller, 1995; Berman *et al.*, 1996). CD31 is localised in the intracellular junctions of endothelial cells (Muller *et al*, 1993) and is crucial to the process of leukocyte transmigration through intercellular junctions of vascular endothelial cells. A monoclonal antibody to CD31, or recombinant soluble CD31, blocks transendothelial migration of monocytes by 70-90% (Muller *et al.*, 1993). Recent studies have shown that CD99, a type I transmembrane protein, also

plays a profound role in the transmigration of neutrophils (Lou *et al.*, 2007). CD99 appears to act at a step downstream of CD31 during diapedesis and blocking both molecules has an additive effect, blocking almost all neutrophil and monocyte transmigration (Lou *et al.*, 2007).

Diapedesis of neutrophils through the endothelium is then followed by migration to the site of infection along a chemotatic gradient (Figure 1.1). The invading microorganisms themselves may produce chemoattractants such as fMLP, involved in recruiting neutrophils to the site of infection. Other chemotactic agents such as IL-8 may be released from the local phagocytes following initial interactions with infectious agents (Hachicha *et al.*, 1998). This allows an amplification of the preliminary inflammatory response, initiating a second wave of neutrophil recruitment.

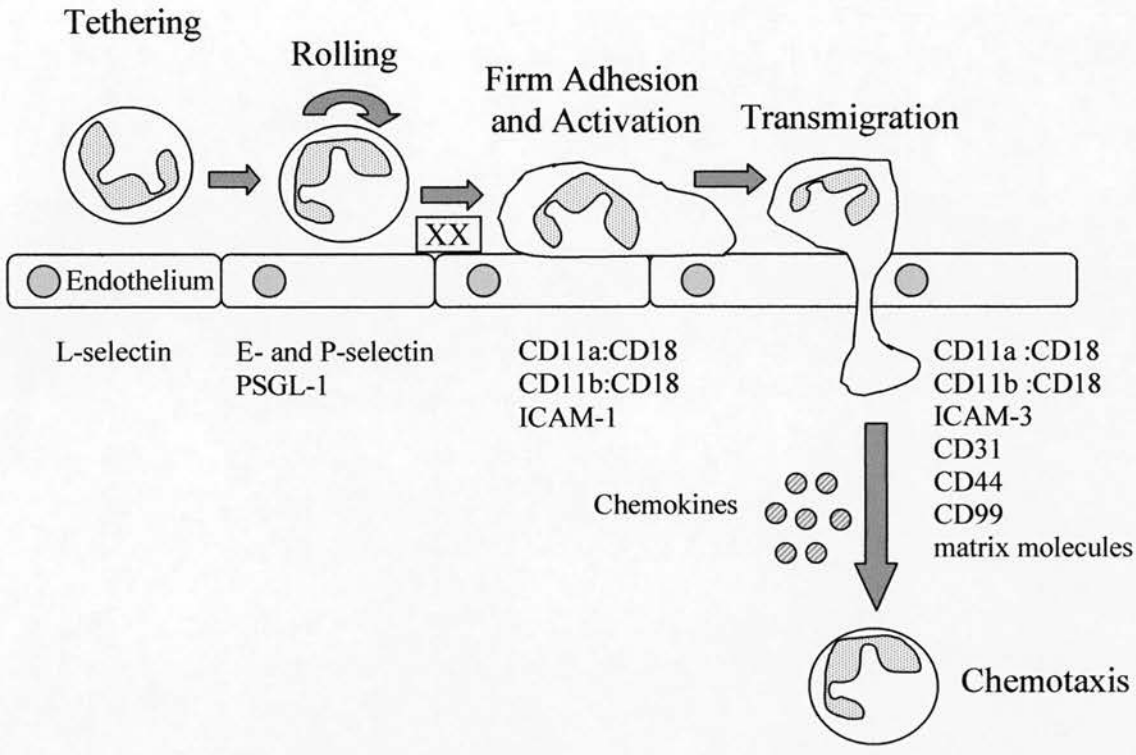


Figure 1.1 Schematic representation of the mechanism involved in neutrophil recruitment

Neutrophil recruitment is controlled by a cascade of multiple molecular interactions. Initial tethering of neutrophils involves reversible binding to the endothelium through selectins on the endothelium and their carbohydrate ligands on the neutrophils. This enables neutrophils to roll along the blood vessel wall and to sense activating factors such as chemokines that are deposited on the endothelial surface. This leads to the activation of neutrophil integrins (CD11a, CD11b) and ICAM-1 which mediate firm adhesion, a prerequisite for directed migration of neutrophils on the endothelial cell surface. Tight binding arrests the rolling and allows the neutrophil to transmigrate across the endothelium and enter the site of infection. Finally, the neutrophil migrates along a concentration gradient of chemokines secreted by the cells at the site of infection. XX = Stop signal.

1.3.1 Phagocytosis

Once a neutrophil has migrated into the tissue, its primary purpose is to recognise and destroy pathogens. Phagocytosis is a process utilised by neutrophils to ingest and clear large particles (>0.5um), including infectious agents and cellular debris. Neutrophils may phagocytose microbes through direct binding of lectins (Sharon and Ofek, 1995). However, in many instances effective phagocytosis and clearance of infectious agents additionally requires the availability of opsonins, to facilitate the adherence of the bacteria and other microbes to opsonin receptors on the surface of the neutrophil. Opsonisation with antibodies or complement components may allow phagocytosis mediated through Fc receptor recognition of the Fc portion of antibodies binding on the bacterial or viral cell surface or via complement receptors binding to C3b (Scribner and Fahrney, 1976). Following recognition and pathogen binding, a phagosome forms containing engulfed micro-organisms. This then fuses with the intracellular granules, allowing the neutrophil to release a variety of antimicrobial agents into a contained microenvironment.

During phagocytosis a substantial increase in oxygen consumption is observed, called the respiratory burst activity, which requires translocation and assembly of the cytosolic components of the NADPH oxidase enzyme system with membrane-bound flavocytochrome, cytochrome b558. This process, through the reduction of oxygen by NADPH oxidase, allows the generation of toxic reactive oxygen intermediates (ROI) such as superoxide anions and via the activity of superoxide dismutase, generation of hydrogen peroxide. Further reduction leads to the production of more toxic oxygen radicals such as hydroxyl radical (OH) from H_2O_2, in a reaction catalysed by Fe^{2+} or the production of hypochlorous acid, through the action of myeloperoxidase. These substances have highly powerful antimicrobial activities, the importance of which is illustrated in chronic granulomatous disease (CGD). CGD is characterized by various genetic defects in essential NADPH oxidase components and reults in an inactive oxidase. Patients with CGD experience severe, recurrent life-threatening bacterial and

fungal infections and often develop granulomas formed by the fusion of monocytes and macrophages that have phagocytosed bacteria, but are unable to destroy them as a result of a defective NADPH oxidase (Heyworth *et al.*, 2003).

1.3.2 Apoptosis

The majority of neutrophils do not migrate from inflamed sites but are cleared locally by neighbouring cells or by macrophages. However, in order for neutrophils to be removed from sites of inflammation they must first undergo apoptosis. Apoptosis is a genetically regulated form of cell death, which is important for normal development and cell turnover in adult organisms. Neutrophil apoptosis is crucial for the successful resolution of inflammation and the balance between neutrophil apoptosis and necrosis in inflamed tissues is an important determinant of the degree of tissue injury. The phagocytic removal of intact, apoptotic neutrophils prevents them from releasing their cytotoxic content into the extracellular milieu that would occur if the cells died by necrosis. Death by apoptosis and safe removal by phagocytic cells thus helps to limit tissue damage during resolution of inflammation. Dysregulation of apoptosis affects many pathological conditions. Accelerated apoptosis is evident in acute and chronic degenerative diseases, immunodeficiency, whereas insufficient apoptosis or defective apoptotic cell clearance can cause cancer and autoimmunity (Fadeel *et al.*, 1999).

When neutrophils become apoptotic, a chain of highly regulated molecular events occur that result in distinct structural, morphological and biochemical phenotypes. Apoptotic neutrophils tend to be smaller but more vacuolated as a result of cytoplasmic changes and the typical multi-lobed nucleus seen in non-apoptotic neutrophils fuses into one or sometimes several distinct, visually disconnected nuclear lobes. The nuclear chromatin condenses into dense, crescent shape structures with the nucleolus becoming more prominent. Endogenous endonuclease activation is responsible for internucleosomal cleavage into characteristic DNA fragments of 180-200 base pairs of the chromatin (Majno and Joris, 1995). Major cell surface changes occur in neutrophils undergoing

apoptosis (Hart *et al.*, 2000). For example, the distribution of plasma membrane (PM) phospholipid changes dramatically. Phosphatidylserine, normally located on the inner leaflet of the PM of non-apoptotic cells, flips onto the external surface of apoptotic cells (Fadok *et al.*, 1998). Neutrophils also shed FcγRIII (CD16) and L-selectin when undergoing apoptosis (Dransfield *et al.*, 1994; Dransfield *et al.*, 1995; Akgul *et al.*, 2001).

Importantly, as neutrophils become apoptotic their ability to respond to agonists (e.g. fMLP) is dramatically reduced so that they are no longer capable of undergoing chemotaxis and degranulation and their phagocytic abilities are impaired (White *et al.*, 1993). *In vitro*, significant numbers of neutrophils become apoptotic within 18 hours of culture, which is coupled to downregulation of neutrophil functions. *In vivo*, this process might limit their destructive potential before their clearance by macrophages (Savill *et al.*, 1989). The execution of the apoptotic pathway in neutrophils is mediated by caspases. Caspases are the central components of the apoptotic response (Shi, 2002), responsible for morphological changes of apoptotic cells. They are a conserved family of enzymes that irreversibly commit the cell to die. Eleven casapases have been found in humans. Caspase-1, 4, 5 and 13 are involved in cytokine activation and inflammation but not in apoptosis. Of the caspases involved in apoptosis, there are two main types: initiator caspases (caspase-2, 8, 9, 10) and effector caspases (caspases-3, 6 and 7). Initiator caspases are the first to be activated and these cleave and activate the effector caspases. The primary function of effector caspases is the cleavage of key proteins that leads to the characteristic features of apoptotic cells (Nicholson, 1999). Having both initiator and effector caspases means apoptosis is tightly regulated as it involves the coordinated action of multiple caspases.

1.4 Regulation of Neutrophil Function

1.4.1 Priming

It is well recognised that there are complex regulatory mechanisms in place to allow neutrophils to respond to infection when required. The magnitude of neutrophil responsiveness to infection or tissue injury may be influenced by several agents which may allow neutrophils to acquire a state of preactivation, allowing enhanced responsiveness to microbial infection termed 'priming'. Priming agents include PAF, cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), tumour necrosis factor-α (TNFα) and bacterial products such as LPS (Pitrak, 1997). For example, exposure of neutrophils to large concentrations of LPS does not induce oxidative burst but will greatly potentiate the oxidative burst in response to another stimulus such as fMLP or C5a (Haslett *et al.*, 1985). This regulatory mechanism of priming is vitally important for generating a more rapid and powerful neutrophil response, following the initial microbial insult (Condliffe *et al.*, 1998). As a consequence, neutrophil functions such as agonist (e.g. fMLP, C5a) induced degranulation (Fittschen *et al.*, 1988), the respiratory burst (Guthrie *et al.*, 1984) and production of lipid mediators (Doerfler *et al.*, 1989; Doerfler *et al.*, 1994) is greatly enhanced. It has become increasingly apparent that regulation or neutrophil function is also determined by the ability of neutrophils to produce a variety of mediators, which can act in an autocrine manner. This is in contrast to previously held beliefs that neutrophils already possess all mediators necessary to participate in the inflammatory response and have little synthetic capacity. However, neutrophils are known to be capable of producing a large variety of inflammatory mediators such as bioactive lipids and cytokines, which can participate and influence the inflammatory response.

Through metabolism of arachadonic acid, neutrophils produce leukotrienes and prostaglandins such as LTB_4 (McColl *et al.*, 1991) and PGE_2 (Tolone *et al.*, 1977). Futhermore they can secrete a wide variety of cytokines such as IL-8 (Cassatella *et al.*,

1992), IL-6 (Cicco *et al.*, 1990), TNFα (Dubravec *et al.*, 1990; Djeu *et al.*, 1990) and IL-1 (Tiku *et al.*, 1986; Scapini *et al.*, 2000). Thus, the role of the neutrophil is not only determined by their ability to respond to agents in the extracellular milieu, but also by their capacity to direct and influence the inflammatory response.

1.4.2 Co-operative Signalling in Neutrophils

An emerging theme in neutrophil adhesion research is that selectin-mediated cell tethering and rolling alone is not sufficient to enable cells to become firmly adherent but is synergistic with signalling via chemokine ligation in activation of integrins to bind adhesion molecules more efficiently under shear flow. Neutrophil activation occurs in response to signalling molecules that are recognized by receptors on their plasma membranes, including members of the G-protein coupled receptors that recognise PAF, TNFα and GM-CSF and others (Ben-Baruch *et al.*, 1995). Traditionally, these chemotactic factors were thought to be supplied by cells outside of the vascular system, at sites relatively distant from the initial contact between endothelial cells and neutrophils. However, it is now known that inflamed endothelial cells express signalling factors for neutrophils. This is a powerful mechanism for spatially localising the activation response of the target neutrophil and one which eliminates the need for the release of signalling factors in solution, which can have deleterious consequences (Zimmerman *et al.*, 1993). For example, PAF can act as a signalling molecule, as PAF is rapidly synthesized by stimulated endothelial cells but its expression on their surfaces is transient; thus there are mechanisms that control its signalling action (Prescott *et al.*, 1984). PAF induces inside-out signalling of β_2-integrins on the neutrophils and other activation responses and blocking the PAF receptor on neutrophils inhibits these events at the endothelial surface (Zimmerman *et al.*, 1990; Lorant *et al.*, 1991). In addition, exogenous PAF induces the relevant signalling events and does so when neutrophils are tethered to P-selectin (Lorant *et al.*, 1993). Engagement of the ligand for P-selectin on neutrophils appears to facilitate inside-out signalling of β_2 integrins (Lorant *et al.*, 1991). The tethering of neutrophils by P-selectin to the surface of the endothelium is known to

be essential in allowing PAF to interact with its receptor and thereby induce neutrophil activation/adhesion. Thus, P-selectin and PAF act in a complementary fashion. Such coordinated action of tethering and signalling factors is likely to be a general mechanism of adhesion-dependent signalling in the vascular system and elsewhere.

PAF initiates signalling cascades in neutrophils that are associated with the endothelial plasma membrane (Zimmerman *et al.*, 1990). Thus, it acts in a juxtacrine fashion (Zimmerman *et al.*, 1993; Lorant *et al.*, 1993; Bosenberg and Massague, 1993). One consequence of juxtacrine activation of neutrophils by PAF is functional upregulation of CD11/CD18 integrins on the PMN. These integrins bind to counter receptors on the EC, enhancing the avidity of adhesion over that provided by P-selectin alone. Juxtacrine signalling adds another level of precision to spatially localised information transfer between cells and is particularly well-suited for inflammatory and vascular interactions, where tightly localised cellular activation is frequently required. In endothelial cell interactions with neutrophils, E-selectin may act as a juxtacrine signalling molecule and IL-8 and other chemokines may signal while localised at the endothelial cell surface (Zimmerman *et al.*, 1993). For example, Ruchaud-Sparagano *et al.* (2000) showed that exposure of neutrophils to soluble E-selectin and PAF in combination induced a synergistic effect upon β_2-integrin-mediated adhesion and demonstrated that E-selectin specifically prolonged elevation of $[Ca^{2+}]_i$ in response to PAF. Thus, combinations of tethering and signalling molecules regulate neutrophil adhesive interactions with EC and the time-dependent expression of different patterns of tethering and signalling molecules by EC provides a general mechanism for differential adhesion and activation. Combinations of such molecules also regulate other functional responses of the neutrophils that are important in inflammation.

1.5 Neutrophil Signalling

Chemokines and chemoattractants are known to initiate several intracellular signal transduction cascades that lead to neutrophil activation and altered functional responses. The main pathways activated are intracellular Ca^{2+} signalling, phospholipid signalling and tyrosine phosphorylation.

1.5.1 Intracellular Ca^{2+} Signalling

Signals for increases of the $[Ca^{2+}]_i$ are generated by chemokine receptors that are activated by chemoattractants such as fMLP and PAF. Chemoattractants alone are known to induce Ca^{2+} mobilization through the release of Ca^{2+} from intracellular stores, which occurs through activation of the GPCR. Receptor activation leads to PLC activation, which cleaves the PM minor phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2) to generate the second messengers inositol (1,4,5)-trisphosphate (IP_3) and DAG. IP_3 then binds to an IP_3 receptor located on the endoplasmic reticulum (ER) and causes the release of Ca^{2+} which floods into the cytoplasmic compartment. When agonists such as PAF bind to a GPCR, the interaction of IP_3 with its receptor in the ER results in rapid Ca^{2+} release from ER stores. This release of Ca^{2+} from intracellular stores can result in a subsequent activation of Ca^{2+} influx across the PM by mechanisms generally referred to as store-operated calcium entry (SOCE), see figure 2.1.1 (Putney, Jr., 1986).

As neutrophils are electrically non-excitable, store-operated Ca^{2+} channels (SOCs) rather than voltage-operated Ca^{2+} channels (VOCCs) provide the major mechanism for Ca^{2+} influx. SOC is defined as a channel that opens in response to depletion of the internal Ca^{2+} stores. SOC is activated when IP_3 discharges Ca^{2+} from intracellular stores in the ER or sarcoplasmic reticulum (SR) (Hoth and Penner, 1992).

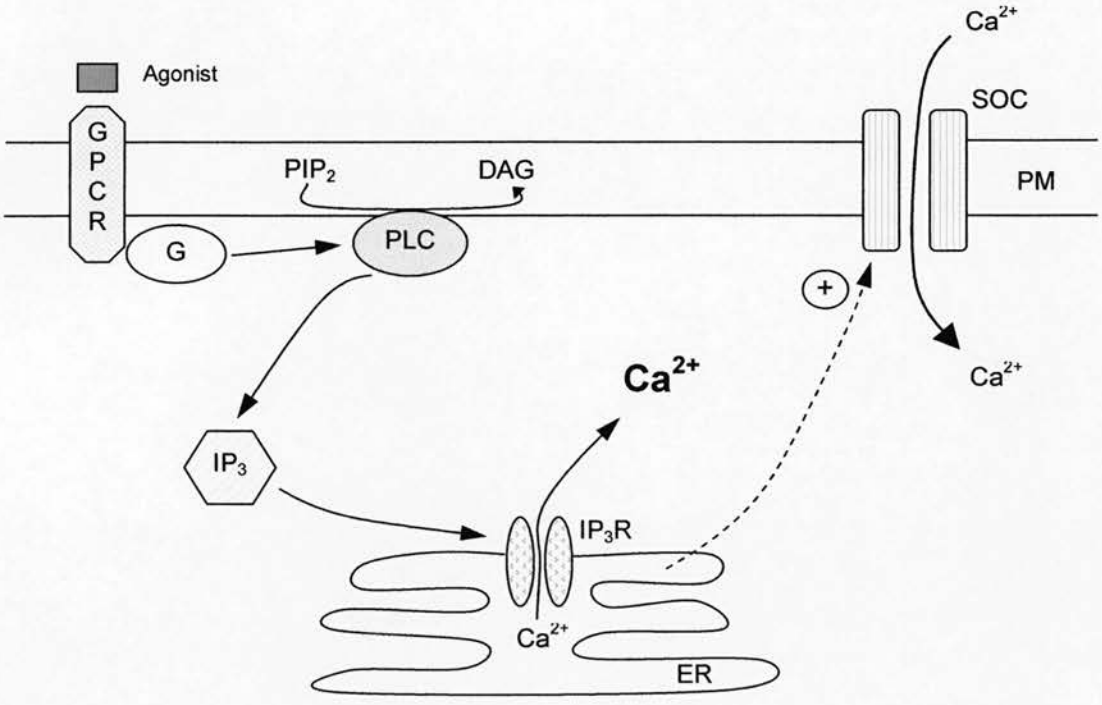


Figure 1.5.1 – Schematic Model of Store Operated Calcium Entry (SOCE)

An agonist binds to G-protein coupled receptor (GPCR) resulting in activation of PLC, which cleaves PIP_2 and generates diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). Binding of IP_3 to its receptor (IP_3R) on the endoplasmic reticulum (ER) causes release of Ca^{2+} into the cytoplasm. Intracellular Ca^{2+} store depletion communicates with store-operated channels (SOC) in the plasma membrane leading to the activation of store-operated Ca^{2+} entry (SOCE). SOCE is Ca^{2+} entry via SOCs.

During the past 20 years, identification and characterization of SOC and evaluation of its function have become one of the key goals of Ca^{2+} research. Recent studies suggest that alterations in the activities of SOCs may be at the heart of several inflammatory diseases (Nilius *et al.*, 2005; Ma *et al.*, 2006). Thus, understanding the regulation of SOCs may prove to be important in developing specific drugs targeted to SOC.

1.5.2 TRPC

The molecular basis of Ca^{2+} influx in neutrophils is not well understood (Li *et al.*, 2002). However, the transient receptor potential (TRP) family of non-voltage activated Ca^{2+} channels are thought to represent a key potential regulator of Ca^{2+} entry in myeloid cells. Overexpression of the TRP family enhanced SOCE, while reduction of TRP expression using antisense strategies decreases SOCE uptake (Zhu *et al.*, 1996; Birnbaumer *et al.*, 1996).

TRP channels have six putative transmembrane spanning regions with a pore domain between regions 5 and 6 and have been subdivided into seven major subgroups based on their amino acid sequence homology; (1) TRPC (classical or canonical), (2) TRPV (vanilloid), (3) TRPM (melastatin), (4) TRPP (polycystin), (5) TRPML (mucolipin), (6) TRPA (ankyrin) and (7) TRPN (NOMPC).

TRP channels are activated by multiple stimuli including intra- and extracellular messengers, ligand activation, receptor activation, chemical, mechanical and osmotic stress (Putney, 2005). Little was known about TRP expression and function in neutrophils until recently. Table 1.5.2 summarises current data relating to the TRPC family in myeloid cells. Several groups have demonstrated that TRPC6 and TRPC3 were present in human neutrophils (Heiner *et al.*, 2003; Itagaki *et al.*, 2004; McMeekin *et al.*, 2006). Itagaki *et al.* (2004) also confirmed that multiple TRPC proteins expressed by human neutrophils participate in SOCE.

SOCE Channel	Proposed Mode(s) of Activation	Expression in Myeloid Cells	Knockout Mouse Model	Disease
TRPC1	PLC, store depletion, mechanical (stretch), receptor operated?	N,M,P	No model reported	Asthma, COPD
TRPC2	PLC, DAG, store deletion?	Pseudogene in humans	Required for neuronal excitability in pheromone signal transduction	N/A
TRPC3	PLC, DAG, OAG, Src TK, IP_3, store depletion	N,M,P	No model reported	Asthma, COPD
TRPC4	PLC, GTPγS, store depletion?	N,P	Impaired endothelium-dependent vasorelaxation & endothelial barrier function	Vascular diseases e.g. hypertension & vascular inflammation
TRPC5	PLC, GTPγS, receptor-operated, store depletion?, Rac, PI3K	M,P	No model reported	Duchenne muscular dystrophy, Hypertension
TRPC6	PLC, DAG,OAG, Src TK, 20-HETE, flufenamate	E,N,M,P	Defective vasomotor control, sensitized myogenic response	Focal segmental glomerulosclerosis & chronic kidney disease
TRPC7	PLC, DAG,OAG, 20-HETE, store depletion	N	No model reported	

Table 1.5.2 Properties of TRPC Channels

E = Eosinophil, N = Neutrophil, M = Monocyte, P = Platelet.

1.5.3 Role of Intracellular Ca^{2+} Signalling

Elevation of $[Ca^{2+}]_i$ is a ubiquitous mechanism of signalling that initiates a wide variety of physiological processes through the activation of enzymes that alter protein function. Important cellular responses in neutrophils that are mediated or essentially regulated by the $[Ca^{2+}]_i$ include production and release of arachidonic acid products (Krump *et al.*, 1995), degranulation (Nusse *et al.*, 1998) and respiratory burst (Thelen *et al.*, 1993; Granfeldt *et al.*, 2002). Ca^{2+} has also been shown to be important for chemotaxis, in particular for activation of β_2-integrins (van *et al.*, 1993) allowing the firm adhesion to the endothelial lining of blood vessels and for the recycling of integrins in migrating neutrophils (Lawson and Maxfield, 1995). These functional responses, which are important for host defence are regulated by exogenous mediators, including cytokines, chemokines, bacterial peptides and growth factors. It is now becoming clear that tight spatio-temporal regulation of Ca^{2+} concentrations following exposure to these mediators represents a key mechanism for initiation and control of neutrophil responses. For example, SOCs can mediated spatially confined Ca^{2+} signals as seen during neutrophil phagocytosis, where Ca^{2+} influx signals have two temporally and spatially separate components associated with phagocytic cup formation and triggering of the oxidative burst respectively (Tian *et al.*, 2004). The concentration of Ca^{2+} inside the cell is required to be finely tuned by a complex interplay between ion channels, transporters, pumps and binding proteins.

Recent exciting reports suggest that disruption of the underlying control mechanisms of SOCE in neutrophils may lead to inappropriate cell activation, contributing to development of major life threatening disease including atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and allergic reactions (Lee *et al.*, 2005; Steinckwich *et al.*, 2007). Several groups have also demonstrated that SOCE is required for the activation of neutrophil functions seen in an inflammatory context (Kuhns *et al.*, 1998; Adams *et al.*, 2001) and clinical data (Hauser *et al.*, 2000) suggests that neutrophil dysregulation following injury and inflammation involves abnormally

enhanced SOCE. Therefore, the pivotal role of Ca^{2+} signalling in the control of neutrophil cell function and their impact on disease processes may also lead to the identification of new therapeutic targets.

1.5.4 Phospholipid Signalling

The interaction of chemoattractants with their GPCR receptor expressed on neutrophils triggers multiple second messengers through the activation of PLC, phospholipase D (PLD) and phospholipase A2 (PLA_2) and rapidly stimulates phosphatidylinositol-3-kinase (PI 3-kinase), as well as activating tyrosine phosphorylation. The second messengers resulting from the GPCR activation act on various intracellular kinases, including PKC and mitogen-activated protein kinases (MAPKs). The activation of these signal transduction pathways is known to be responsible for various biochemical responses which contribute to physiological defence against bacterial infection and cell disruption.

Fundamental studies in developmental biology, cellular biology and immunology have established critical roles for PI 3-kinase signal transduction pathways in cellular chemotactic responses, proliferation, apoptosis and survival. Structurally, PI 3-kinases exist as heterodimeric complexes, consisting of a p110 catalytic (classified α, β, γ, and δ) and a p55, p85, or p101 regulatory subunit (Vanhaesebroeck *et al.*, 1997; Wymann and Pirola, 1998). These enzymes can be further divided into 2 subclasses; class 1A (PI3-kinase α, β and δ) and class 1B (PI 3-kinase γ) on the basis of their mechanism of activation. Class 1A PI 3-kinases are stimulated by recruitment of regulatory subunits to activated growth factor, cytokine and antigen receptors, while Class 1B is stimulated by direct binding of the catalytic subunit to activated GPCRs, such as chemokine receptors (Okkenhaug and Vanhaesebroeck, 2003; Wymann and Marone, 2005). Activation of both Class 1A and 1B PI 3-kinases may be facilitated by catalytic subunit association with activated Ras family GTPases. The resulting generation of phosphorylated lipid

metabolites recruits and stimulates proteins containing PH (pleckstrin homology) domains.

PI 3-kinases are known to play a pivotal role in the ability of neutrophils to undergo chemotaxis, as the lipid products they generate, such as PIP_3, are critical for promoting asymmetric F-actin synthesis and thus cell polarization (Wymann *et al.*, 2000; Rickert et al., 2000; Fruman and Cantley, 2002). Their function, however, is not limited to directed migration, as they are also required for phagocytosis and the ability of neutrophils to generate oxygen radicals in response to chemoattractants such as fMLP (Wymann and Arcaro, 1994; Ninomiya *et al.*, 1994; Sasaki *et al.*, 2000b; Cadwallader *et al.*, 2002). The ability of PI 3-kinases to regulate these processes relies on PIP_3-mediated recruitment of two lipid-binding protein kinases, phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B/Akt, both of which can interact with this phosphoinositide derivative via their pleckstrin homology domains. Association of these kinases with PIP_3 at the plasma membrane brings them into close proximity, facilitating the phosphorylation and activation of Akt by PDK1 (Cantley, 2002). PDK1, which is thought to be constituently active, phosphorylates Akt at T308, leading to stabilization of the activation loop in an active conformation. T308 phosphorylation is a requirement for the kinase activation, but phosphorylation of the residue located at the hydrophobic C-terminal region is also required for full activation of the kinase. This Akt S473 kinase (PDK2) has not been identified as yet; however, findings from several recent studies suggest a role for protein kinase integrin-linked kinase (ILK) in the activation process, but it remains unknown whether or not ILK directly phosphorylates Akt at S473 (Persad *et al.*, 2001; Viniegra *et al.*, 2005). These proteins are, in turn, responsible for many of the downstream signalling events associated with PI 3-kinase activity.

Neutrophils express all 4 members of PI 3-kinases and their involvement in cell migration is supported by the ability of nonselective PI 3-kinase inhibitors, such as LY294002 and wortmannin, to mitigate neutrophil chemotaxis. Moreover, demonstration that inhibition of a specific isoform of these lipid kinases may alter

neutrophil extravasation into inflamed tissues has been suggested by the partial reduction in chemoattractant-directed migration of these cells in mice deficient in the p110 catalytic subunit (Hirsch *et al.*, 2000; Li *et al.*, 2000; Sasaki *et al.*, 2000a; Hannigan *et al.*, 2002). Thus, these observations have triggered considerable interest in the development of specific small molecule inhibitors to PI 3-kinases that may prevent undesirable neutrophil accumulation in certain inflammatory disease states in humans (Stein and Waterfield, 2000).

1.5.5 Tyrosine Phosphorylation

An increase in protein tyrosine phosphorylation is one of the earliest events after stimulation of neutrophils, with a wide variety of stimuli inducing activation of selective cell responses. Many studies have shown that neutrophil responses to chemoattractants can be blocked with inhibitors of tyrosine phosphorylation, proving that tyrosine phosphorylation plays an important role in chemoattractant signalling (Welch *et al.*, 1996). The Src family tyrosine kinases have been implicated in a number of GPCR signalling pathways in leukocytes. Stimulation of many GPCRs directly increases the enzymatic activity of Src family kinases (Ptasznik *et al.*, 1995; Luttrell *et al.*, 1996). In neutrophils, the Src family kinases have been implicated in fMLP signalling as treatment of neutrophils with Src family kinase inhibitors or use of cells from $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mutant mice blocks degranulation responses to this agonist (Mocsai *et al.*, 1999; Mocsai *et al.*, 2000). Together, these reports would suggest that the overall dominant role for Src family kinases, and specifically for Src family members in neutrophils, is a positive function in which the kinases propagate downstream signalling pathways after chemokine stimulation.

1.5.6 Tyrosine Kinases in Signalling by Adhesion

As described earlier (1.3) adhesion to endothelial counter-receptors represents an essential requirement for neutrophil transmigration into inflamed tissues. Additionally,

neutrophil interaction with extracellular matrix proteins or tissue cells regulates their secretion of proinflammatory and toxic compounds. Signalling by both selectins and integrins plays a critical role in neutrophil functions and has been demonstrated to involve tyrosine kinases. The signalling capacity of neutrophil L-selectin (Laudanna *et al.*, 1994) is blocked by tyrosine kinase inhibitors (Waddell *et al.*, 1995). Additionally, engagement of the selectin ligand PSGL-1 triggers tyrosine phosphorylation and tyrosine kinase–dependent interleukin-8 secretion by neutrophils (Hidari *et al.*, 1997). Integrin signalling is also strictly dependent on tyrosine kinases in neutrophil and macrophages, and substantial progress has been made in the identification of the tyrosine kinases that are implicated in integrin signal transduction (Berton and Lowell, 1999).

Tyrosine kinases implicated so far in integrin signaling in neutrophils are Src-family tyrosine kinases (Berton *et al.*, 1994; Yan *et al.*, 1995; Lowell *et al.*, 1996), spleen tyrosine kinase (Syk) (Yan *et al.*, 1997; Fernandez and Suchard, 1998), and focal adhesion kinase (Fak) (Fernandez *et al.*, 1997). Studies using mice deficient in the major Src family kinases present in neutrophils have demonstrated that the primary positive signalling role for these enzymes is in the integrin pathway (Lowell and Berton, 1999; Lowell, 2004). Deficiency of Hck and Fgr renders neutrophils and macrophages nonresponsive to adhesion-mediated activation after crosslinking of β_1, β_2, or β_3 integrins.

1.6 Selectins and their Receptors

Studies *in vitro* and *in vivo* have revealed the critical role of the selectin family of molecules in the initial capture and subsequent rolling on vascular endothelial ligands (Lawrence and Springer, 1991) before their firm adhesion and diapedesis at sites to tissue injury and inflammation. The selectins are three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin) and vascular endothelium (E & P-selectin). Each receptor contains an amino-terminal domain related to those in Ca^{2+}-dependent (C-type) lectins, followed by an epidermal

growth factor (EGF)-like motif, a series of consensus repeats similar to those in complement-regulatory proteins, a transmembrane domain and a cytoplasmic tail. The lectin domain is directly involved in mediating cell-cell contact through Ca^{2+}-dependent interactions with cell-surface carbohydrates (Bevilacqua and Nelson, 1993; Bouyain *et al.*, 2001).

With respect to neutrophil recruitment at sites of inflammation, an emerging generalization is that P and L-selectin function in the earliest phases of acute response, whereas E- and possibly L-selectin are critical during later stages (Rosen and Bertozzi, 1994; Ley *et al.*, 1995). Much recent investigation has focused on the identification and characterization of glycoconjugate ligands for the selectins (Rosen and Bertozzi, 1994; Sperandio *et al.*, 2006). In particular, it has been demonstrated that the tetrasaccharide sialyl Lewis x (sLe^x) and related terminal sugars expressed by neutrophils, monocytes and some lymphocytes can act as ligands for E-selectin. sLe^x is also recognised by P-selectin and L-selectin, although affinity of binding may differ substantially (Bevilacqua and Nelson, 1993; Sperandio *et al.*, 2006). These findings have been confirmed in studies of leukocyte recruitment in mice deficient in both E- and P-selectins (Frenette *et al.*, 1996; Kakkar and Lefer, 2004) and in patients with leukocyte adhesion deficiency syndrome type II (LADII) who lack the ability to produce appropriately glycosylated selectin ligands (Phillips *et al.*, 1995; Hidalgo *et al.*, 2003).

The vascular selectins are recognized by neutrophil receptors which carry the carbohydrate ligand for the selectins, sLe^x. The best characterized selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1) which is found on leukocytes and platelets. PSGL-1 binds to P-, E- and L-selectin *in vitro* and represents an important functional ligand for all of these molecules. P-selectin appears specifically to recognize PSGL-1 whereas E-selectin recognizes a much wider range of sLe^x-carrying proteins and lipids. For example, the carcinoembryonic antigen (CEA) subfamily of the Ig superfamily have been implicated in recognition and as adhesion-supporting molecules and for neutrophils CD66 antigens have been shown to mediate adhesion to E-selectin (Kuijpers *et al.*,

1992). Recently there have also been reports that CD44 could bind to E-selectin (Dimitroff *et al.*, 2001) and that CD44 is a physiological E-selectin ligand on mature human and mouse myeloid cells (Katayama *et al.*, 2005). HCELL, which is a specialised sialofucosylated glycoform of CD44, is highly expressed on neutrophils and is also known to be a potent E-selectin ligand (Dimitroff *et al.*, 2001; Dagia *et al.*, 2006). One potential PSGL-1 independent ligand for E-selectin is E-selectin ligand-1 (ESL-1), an N-glycosylated protein that may be oligomerized to enhance binding avidity, compensating for its relatively low binding (Xia *et al.*, 2002). ESL-1 binds specifically to E-selectin but not to P-selectin (Levinovitz *et al.*, 1993) and is expressed by a wide variety of cell types, although the glycoform that binds to E-selectin has only been found in myeloid cells (Vestweber and Blanks, 1999). ESL-1 has been described in murine leukocytes but its function and expression in human cells is uncertain.

The question of how the selectins achieve high affinity and selectivity *in vivo* has caused tremendous interest. Affinity, as well as selectivity, could also derive from distinctive features of the carbohydrate chains or the protein core. There is growing evidence indicating that sulphation is a critical feature for at least two classes of selectin ligands, L- and P-selectin (Feizi, 2001). Two enzymes catalyzing carbohydrate sulphation N-acetylglucosamine 6-O-sulfotransferase [(GlcNAc6ST)-1 and -2)] were found to be involved in the generation of 6-sulfo sLe^x which is important for L-selectin ligand activity on HEV (Uchimura *et al.*, 2005; Kawashima *et al.*, 2005). In the case of P-selectin, early experiments with selenate (a salt of selenic acid and a metabolic inhibitor of sulphation) suggested the potential importance of sulphation for neutrophil ligands (Aruffo *et al.*, 1991). Furthermore, sulphation of tyrosine residues at the N-terminus of PSGL-1 has been reported to significantly influence binding of P-selectin to PSGL-1 (Ramachandran *et al.*, 1999). Thus, both L- and P-selectin recognise sulphated ligands. In addition both L- and P- but not E-selectin can bind sulphated molecules such as heparin and sulfatides (Norgard-Sumnicht *et al.*, 1993). Therefore, sulphation is important in L- and P-selectin ligand interactions but E-selectin recognition of ligands appears to differ from these two classes of selectins. These findings suggest that each

selectin preferentially interacts with very specific ligand structures. Although selectins bind with moderate affinity to sialylated, fucosylated and (in some cases) sulphated carbohydrate ligands, typified by the sLe^x, preferred natural ligands are more complex glycoconjugates. If neutrophils use multiple receptors to bind selectins, this may provide an opportunity to selectively inhibit specific effects of selectins upon neutrophil function.

1.6.1 Regulation of Selectins

As contact formation between most leukocytes and endothelium is initiated by selectins, the regulation of their presence on the cell surface is important for the control of leukocyte extravasation. The two endothelial selectins, E- and P-selectin, are absent from the cell surface of non-activated endothelial cells and become induced upon exposure of the endothelium to various pro-inflammatory stimuli. This ensures that these selectins are only present on endothelium in proximity to inflamed tissues. L-selectin in contrast, is constitutively expressed on leukocytes, but is shed by proteolytic cleavage following cellular activation (Bevilacqua and Nelson, 1993). Proteolytic shedding occurs on neutrophils within minutes and can be induced by a variety of chemoattractants and activating factors such as C5a, fMLP, LTB_4, IL-8, TNFα, granulocyte-macrophage colony stimulating factor (GM-CSF), and calcium ionophores (Griffin *et al.*, 1990). Thus, the function of L-selectin in the initiation of leukocyte-endothelial contacts in inflamed areas is controlled by the regulated appearance of its ligand(s).

Temporal differences in E- and P-selectin expression on vascular endothelium observed in response to tissue injury indicate distinct roles for these molecules in the recruitment of leukocytes. E-selectin expression is limited to endothelium and principally to endothelium in response to inflammatory stimuli such as interleukin-1 (IL-1), TNF-α or bacterial LPS. The loss of E-selectin from the surface of activated endothelial cells is most likely caused by a combination of factors. Firstly, E-selectin gene transcription is

sharply downregulated within 6 to 9 hours after induction (Bevilacqua *et al.*, 1989) and the E-selectin mRNA has a short half life (Ghersa *et al.*, 1992). Secondly, E-selectin is rapidly internalized and degraded in lysosomes (Subramaniam *et al.*, 1993). Endothelial cells have also been shown to shed E-selectin into the microcirculation following *in vitro* activation (Pigott *et al.*, 1992; Newman *et al.*, 1993). The combination of these processes collectively ensures that the expression of E-selectin at the surface of cytokine-stimulated endothelium is transient and the activity of the selectins is likely to be controlled in large part by regulation of their appearance and disappearance from the cell surface.

1.6.2 Selectins and Disease

The neutrophil plays an essential role in host defence however there is accumulating evidence that dysregulation of neutrophil function can result in injury to healthy tissue and is associated with the pathogenesis of a large number of inflammatory conditions. In many diseases such as rheumatoid arthritis, vasculitis and glomerulonephritis, the neutrophil is thought to contribute to disease progression (Weissmann and Korchak, 1984; Heinzelmann *et al.*, 1999). In acute respiratory distress syndrome (ARDS), neutrophils may cause injury through enhanced production of reactive oxygen species (McGuire *et al.*, 1982). Influx of neutrophils and subsequent activation is also thought to be involved in ischemia-reperfusion injury (Jordan *et al.*, 1999).

The inappropriate or abnormal sequestration of neutrophils at specific sites is a central component in the development of a variety of autoimmune diseases and pathologic inflammatory disorders. Therefore, therapeutic agents that block neutrophil-endothelial interactions have a dramatic effect on the progression of inflammatory responses in a number of animal models (Albelda *et al.*, 1994). The restricted role of the selectins in directing leukocyte interactions with vascular endothelium makes these molecules extremely attractive targets for intervention by therapeutic agents (Etzioni, 1994).

The function of selectins *in vivo*, as well as their expression patterns *in vivo*, points to their involvement in a wide variety of human diseases including atherosclerosis, ischemia-reperfusion injury, acute lung injury, rheumatoid arthritis, graft rejection and various cancers. E-selectin has been shown to be expressed on activated endothelium at sites of active inflammation (Panes *et al.*, 1999). In animal models of acute lung injury, antibodies against E-selectin were protective against lung damage induced by several stimulants (Mulligan *et al.*, 1993). In a primate model of extrinsic asthma, antibody blocking data has suggested that E-selectin plays a major role in neutrophil influx associated with late phase airway obstruction (Gundel *et al.*, 1991). In animals sensitive to antigen, a single inhalation exposure induced the rapid expression of E-selectin and exclusively on vascular endothelium that correlated with influx of neutrophils into the lungs and the onset of late-phase airway obstruction. A monoclonal antibody to E-selectin blocked both the influx and the late-phase airway obstruction. The authors concluded that in primates the late phase response was due to E-selectin dependent neutrophil influx and that regulation of E-selectin expression may provide a novel approach to control the acute inflammatory response in diseases such as asthma. Recent data suggest that E- and P-selectin may participate in neutrophil mediated damage associated with ischemia reperfusion injury to the heart (Kakkar and Lefer, 2004). It has also been demonstrated that E-selectin is upregulated in the mouse myocardium following ischemia reperfusion (Jones *et al.*, 2000) and that genetic deficiency of E-selectin conferred cardioprotection with a reduction in myocardial infarct size owing to attenuation of neutrophil accumulation (Sligh, Jr. *et al.*, 1993). Selectin-carbohydrate interactions are also being studied in relation to hematogenous metastasis of cancer cells. E-selectin has been shown to support the adhesion of human colon and breast cancers (Tozeren *et al.*, 1995; Gout *et al.*, 2006). sLe^a and sLe^x are the representative oligosaccharides involved in E-selectin binding both of which are expressed in abundance in human colon and breast cancers (Kim and Varki, 1997).

Elevated plasma levels of soluble E-selectin are associated with various chronic inflammatory conditions. For example, soluble E-selectin levels were found to be

increased in ARDS patients (Ruchaud-Sparagano *et al.*, 1998) and elevated soluble E-selectin levels have been detected in burn patients (Ljunghusen *et al.*, 1996) and breast and gastric cancer patients (O'Hanlon *et al.*, 2002; Alexiou *et al.*, 2003). Levels of soluble E-selectin have also been reported to be significantly higher in rheumatoid arthritis and systemic sclerosis patients (Ates *et al.*, 2004). Soluble E-selectin levels have also shown marked elevations in sepsis, in one study a 20 fold increase over normal range was reported (Newman *et al.*, 1993). Soluble E-selectin appears to correlate with disease severity and/or outcome in several of these diseases as higher levels of persistent elevation are often associated with greater mortality (Gearing and Newman, 1993). Therefore, serum levels of E-selectin may provide a useful additional marker for disease activity or disease severity in patients with inflammatory diseases and measurement of these selectins may increase our understanding of the relationship between *in vivo* endothelial activation and inflammation.

Based on studies like these many laboratories are trying to identify novel anti-inflammatory compounds. These efforts involve antibody-, peptide-, and carbohydrate-based approaches to block selectin-dependent adhesion directly. Selectin antagonists may prove to be effective therapeutics agents alone or may complement drugs designed to block the expression and the function of other adhesion molecules.

Aims

The primary aim of this thesis was to investigate **the nature of the intracellular signals generated following exposure of neutrophils to soluble E-selectin**. Soluble E-selectin levels are elevated in many chronic inflammatory conditions and soluble E-selectin has been shown to promote neutrophil destructive function and potentially influence the progression of inflammation. Therefore, understanding the mechanisms by which selectins influence intracellular signalling pathways could reveal new therapeutic targets for the treatment of inflammatory disease.

Specifically, I wished to:

1. Define the signalling pathways involved in E-selectin-mediated prolongation of intracellular calcium levels and identify the calcium channel involved.

2. Determine the mechanisms involved in the regulation of E-selectin-mediated store operated calcium entry.

3. Study the functional consequence of E-selectin-mediated store operated calcium entry.

4. Identify the glycoprotein ligands for E-selectin on neutrophils that mediated altered store operated calcium entry.

Chapter 2 - Materials and Methods

2.1 Antibodies and other reagents

Reagents

Reagents were obtained from Sigma-Aldrich (www.sigmaaldrich.com) unless otherwise stated. Hanks' balanced salt solution (HBSS) was obtained from Invitrogen (www.invitrogen.com.). Dextran T500 was purchased from Amersham Pharmacia Biotech (www1.amershambiosciences.com). Express-five serum free media was obtained from Invitrogen and High Five cells were a kind gift from Dr Gavin Nicoll, Edinburgh University. Recombinant E-selectin was obtained from R&D Systems (www.rndsystems.com). MRS 1845 and ruthenium red were purchased from Tocris (www.tocris.com). PAF, OAG, thapsigargin, U73122, U73343, LY294002 hydrochloride, LY303511 Fura2-AM, PP2, PP3, SB203580, SB202474, PD98059, 2-APB, R59949 and Ro-31-8220 were purchased from Calbiochem (www.merckbiosciences.co.uk). EZ-Link, Sulfo-NHS-Biotin Reagent was obtained from Pierce (www.piercenet.com). Streptavidin horseradish peroxidise was purchased from Dako (www.dako.com). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. All PCR reagents were purchased from Promega (www.promega.com) unless otherwise stated.

Antibodies

All antibodies were mouse monocolonal IgG unless otherwise stated. Mouse anti-human CD16:RPE and mouse anti-human CD162 clones PL-1 and PL-2 were obtained from Serotec (www.serotec.com). Mouse anti-human E-selectin $F(ab)_2$ (ENA2) was purchased from Abcam (www.abcam.com). Rabbit polyclonal anti-TRPC3 and TRPC6 were purchased from Alamone Labs (www.alomone.com). Rabbit polyclonal IgG anti-phospho-Src family (Tyr416) and rabbit phospho-(Ser) PKC substrate antibody were purchased from Cell Signalling (www.cellsignal.com) and anti-phospho-Akt1/PKBα (Ser473), clone 11E6 was obtained from Upstate (www.upstate.com). PKC sampler kit

(DGKθ, PKCα, β, γ, δ, ε, η, θ, ι, λ & RACK1) was obtained from BD Biosciences (www.bdbiosciences.com). LFA-1 antibody was a kind gift from Dr JE Hildreth. Monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich. Polyclonal goat anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibodies were obtained from Dako (www.dako.com).

2.2 Mononuclear and Polymorphonuclear Leukocyte Isolation

Human peripheral blood was taken from healthy volunteers and collected into tubes containing 4ml of 3.8% sodium citrate (Phoenix Pharmaceuticals Ltd., Gloucester, UK) to a final volume of 40ml and then centrifuged at 350 x g for 20 min. All steps were carried out at room temperature. The resulting upper platelet rich plasma (PRP) layer was removed and autologous serum was prepared by adding 10ml of PRP to 220µl of 1M $CaCl_2$ and incubated for 1 hour (h) at 37°C in glass serum tubes. Leukocytes were separated from erythrocytes in the cell pellet by dextran sedimentation using 2.5ml of 6% (w/v) dextran T500 per 10ml of packed cells, made up to 50ml with 0.9% saline and then left for 30 min at room temperature to sediment. The resulting leukocyte-rich upper layer was then collected and centrifuged at 350 x g for 6 min to pellet the leukocytes. Polymorphonuclear leukocytes were then separated from mononuclear leukocytes using discontinuous isotonic Percoll gradients. Percoll was made isotonic by preparing a 90% solution using 10x PBS. The isotonic Percoll was then used to make 81%, 68% and 55% layers using 1x PBS without $CaCl_2$ or $MgCl_2$. Gradients were prepared by layering 3ml of 68% onto 3ml or 81% Percoll. The cell pellets were then mixed with 3ml of 55% layer and layered onto the gradient. The Percoll gradients were then centrifuged at 720x g for 20 min. Polymorphonuclear leukocytes and mononuclear leukocytes were harvested from the 68%/81% and the 55%/68% interfaces, respectively. Leukocytes were then washed twice in PBS without $CaCl_2$ and $MgCl_2$ before cell culture.

2.3 Quality Control for Cell Isolation

Cells collected from each layer of the gradient were subject to flow cytometry analysis by forward and side scatter (Figure 2.3.1). The PMN fraction was labelled with mouse anti-human CD16:RPE and analyzed using the FL-2 detector, to check the percentage of neutrophils (Figure 2.32). The percentage eosinophils were checked by cytopsin. The PMN used in all experiments were >95% neutrophils, with less than 5% eosinophils present and are hereafter referred to as neutrophils.

2.4 Neutrophil Culture

Neutrophils were resuspended at $4x10^6$/ml in Iscove's DMEM + 10% autologous serum and incubated for 20 h at 37°C and 5% CO_2 in 96 well flat bottom flexible plates (www.bdbiosciences.com) to induce apoptosis.

2.5 Characterisation of Neutrophil Apoptosis by Flow Cytometry

Following *in vitro* culture, neutrophils were labelled with FITC-conjugated annexin V and propidium iodide (PI). Annexin V was diluted 1/500 in annexin V buffer (Ca^{2+} and Mg^{2+} free PBS + 5mM $CaCl_2$) and 280µl was then added to a tube containing 20µl of neutrophils of $4x10^6$/ml and incubated on ice for 10 min. Immediately prior to analysing the sample, 1µl of 1mg/ml PI was added to the cells. Annexin V and PI binding were measured using FL1 and FL2 detectors respectively.

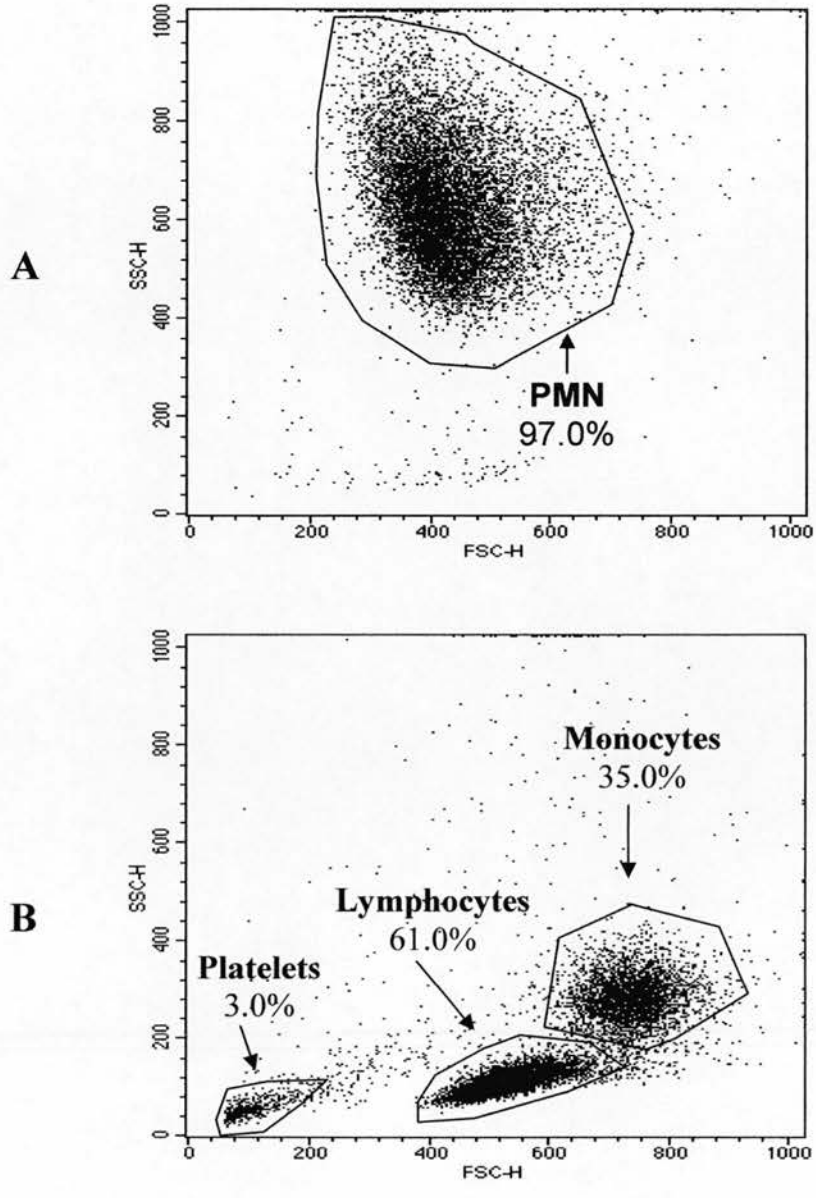


Figure 2.3.1 Flow Cytometry profiles for PMN and monocyte-rich layers Percoll gradient isolation.

Mononuclear and polymorphonuclear (PMN) cells were isolated from peripheral blood and separated by Percoll gradients. Representative flow cytometry FS and SS profiles are shown for (A) the PMN fraction and (B) the mononuclear cell fraction. Gates were drawn to provide an estimate of the percentage of cells corresponding to different leukocyte subpopulations.

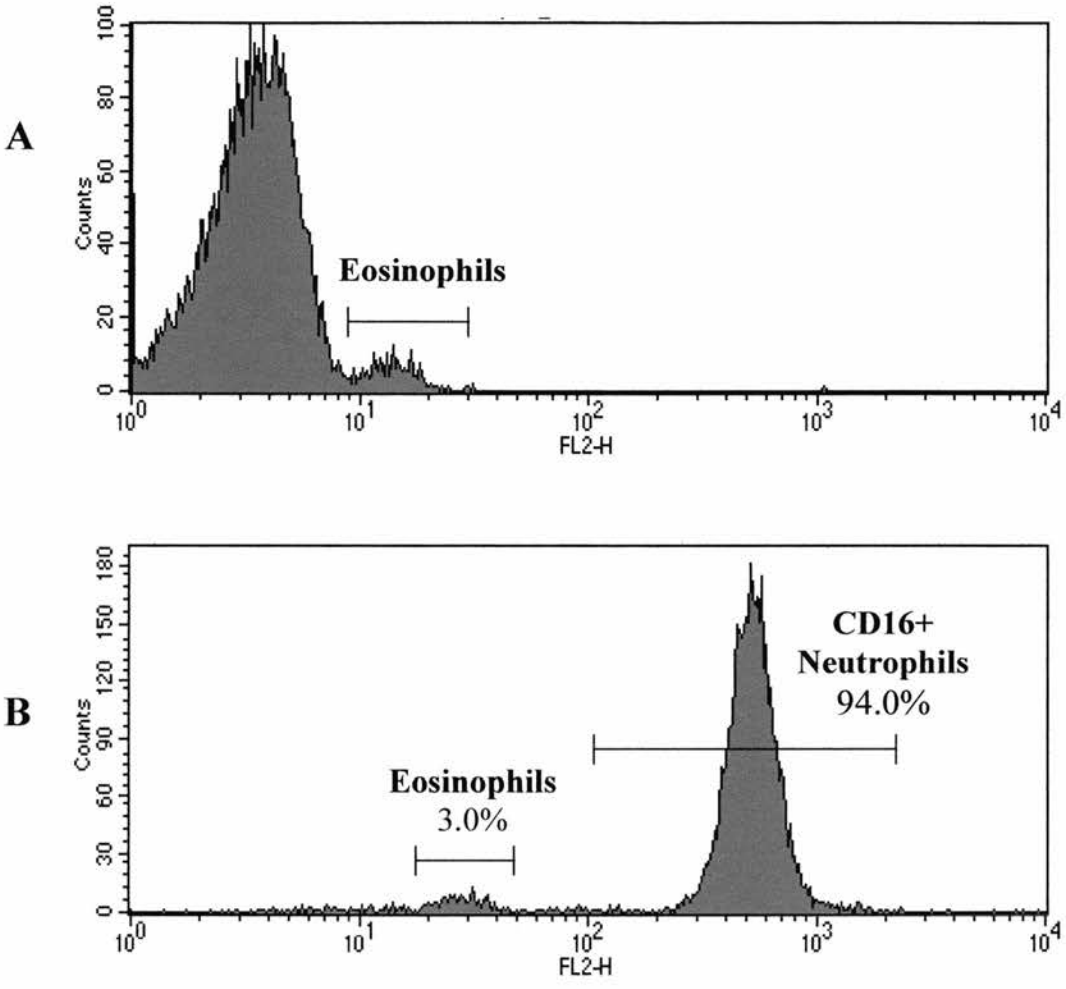


Figure 2.3.2 Flow Cytometry histograms for unlabelled and CD16 labelled PMN's

Fc(gamma)RIIIb (CD16) is a glycosyl phosphatidylinositol (GPI) anchored low-affinity IgG receptor, expressed by human neutrophils. Representative histogram plots are shown for (A) unlabelled PMN fraction showing that eosinophils present in the cell preparation have an increased autofluoresecence compared with neutrophils and (B) CD16 labelled PMN fraction.

2.6 Measurement of $[Ca^{2+}]_i$

Freshly isolated neutrophils were resuspended at 10^7/ml in HBSS (Ca^{2+} and Mg^{2+} free), and were incubated with 2μM Fura2-AM at 37°C for 30 min in the dark. The cells were then washed twice to remove extracellular Fura2-AM and resuspended at 4 x 10^7/ml in HBSS (containing Ca^{2+} and Mg^{2+}). Intracellular calcium was monitored by Fura-2 emission fluorescence at 510nm using 340/380nm dual wavelength excitation in a Perkin Elmer luminescence spectrometer at 37°C with constant stirring. $[Ca^{2+}]_i$ was calculated from the 340/380nm fluorescence ratio as described (Grynkiewicz *et al.*, 1985) using the equation: $[Ca2+]_i = K_d \times B \times [(r - r_{min})/(r_{max} - r)]$, where r is the measured Fura-2 fluorescence ratio between 340 and 380 nm. r_{max}, the maximum 340/380 nm ratio, was obtained by lysing cells with 100μl 0.1% (v/v) Triton X100. r_{min}, the minimum 340/380 ratio, was obtained by adding 10mM EGTA. K_d is the dissociation constant of the Fura-2/Ca^{2+} complex (224 nM), and B is the ratio of fluorescence at 380 nm at 0 and saturating Ca^{2+} concentration (Figure 2.6.1).

2.7 Superoxide Assays

Determination of release of superoxide anions by freshly isolated neutrophils was performed as described previously (Condliffe et al., 1996). Briefly, neutrophils were preincubated with inhibitors as described in figure legends prior to addition of TNF or PAF, in the presence of cytochrome c (1 mg/ml), for 15 min at 37°C. Following this, the reaction was stopped by centrifugation (300 g, 5 min, 4°C) and the superoxide dismutase-inhibitable reduction of cytochrome c was determined for each supernatant by measuring the peak absorbance between 535 and 635 nm using a scanning spectrophotometer. Results are expressed as nanomoles of superoxide anions generated per 10^6 neutrophils.

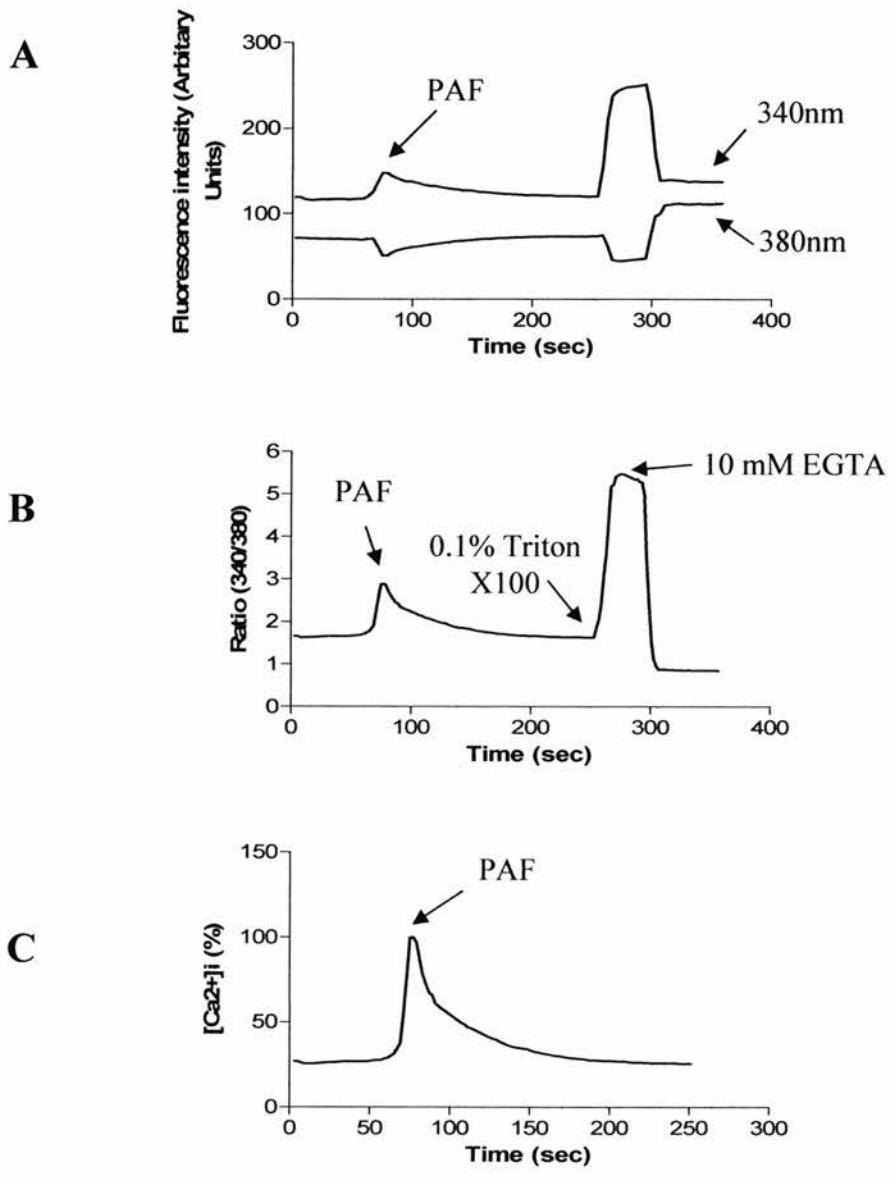


Figure 2.6.1 Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was monitored by FURA-2 emission fluorescence at 510nm using dual 340/380 wavelength excitation (A). Calibration was performed after each experiment using 0.1% Triton X100 to obtain the R_{max} and 10mM EGTA to calculate R_{min} (B). $[Ca^{2+}]_i$ was calculated from the 340/380nm fluorescence ratio and (C) shows a representative calcium trace showing the changes in $[Ca^{2+}]_i$.

2.8 Adhesion Assays

Recombinant E- and P-selectin and fetal calf serum (FCS) in 0.1M bicarbonate (pH10.0) were absorbed to 96 well plates (CoStar) at a concentration of 1μg/ml overnight at 4°C. FCS was heat-inactivated by heating it at 56°C for 30 min. Plates were then blocked with 10% (v/v) heat-inactivated fetal calf serum (HI-FCS) in HEPES-buffered saline for 2 h at 4°C before use in adhesion assays. For adhesion blockade using CD62E, wells were pre-incubated with 1:50 dilution of ENA2 $F(ab)_2$ for 30 min at 4°C. Neutrophils were resuspended at 3 x 10^6/ml in HEPES-buffered saline containing 4mM $CaCl_2$ (assay medium) and were incubated (100μl per well) at 4°C for 30 min with gentle agitation using a rocking platform. Plates were then washed six times in assay medium alone and fixed in 3% paraformaldehyde. Adhesion of neutrophils was viewed under a Zeiss Axiovert microscope, with images captured using AppleMac OpenLab image capture system.

2.9 Migration Assays

Neutrophil chemotaxis was measured using the sub-agarose methods (Chenoweth 1991) with some modifications. Clean glass microscope slides were dipped in 0.5% gelatine in PBS, rinsed and then dried. 5ml of molten 1% agarose (Indubiose) /0.25% gelatine in medium 199 supplemented with 1% BSA was then slowly poured using a 5ml pipette onto the slide and was finally cut with a pattern-former giving one set of three wells per slide. The neutrophils (8μl at 5 x 10^7/ml in medium 199) were added to the central wells and 8μl aliquots of medium 199 plus or minus fMLP/PAF (final concentration 10^{-7}M,) were added to the outer well. The slides were incubated for 2h at 37°C, fixed overnight in 2.5% glutaraldehyde and stained with Diff-Quik Staining set (www.baxterhealthcare.co.uk). Migration distances of duplicate samples were measured by determining the leading edge of neutrophil mobility for both directed and random migration using a Zeiss Axiovert s100 with 10x magnification. Chemotactic response was defined as the specific migration towards the chemoattractant fMLP or PAF.

2.10 Electrophysiology

Before electrophysiological recording the neutrophils were plated onto glass coverslips coated with Sigmacote^{TM}. Neutrophils were plated directly onto clean coverslips and allowed to adhere for 5–10 min.

Human neutrophils were visualized using a water immersion objective (40×, Carl Zeiss Ltd, Welwyn Garden City, UK) and Nomarski differential interference contrast optics. Recording pipettes were made from thin-walled borosilicate glass capillaries (GC150TF, www.harvardapparatus.com) using a patch-pipette puller (Narishige PP-83, www.intracel.co.uk). The patch-pipette was coated with sylgard to reduce capacitance.

For whole-cell voltage clamp experiments, the standard extracellular bath solution contained 140mM NaCl, 1.2mM $MgCl_2$, 1.2mM $CaCl_2$, 5mM KCl and 10mM Hepes; the pH was adjusted to 7.4 using KOH. The pipette solution contained 145mM caesium glutamate, 8mM NaCl, 2mM $MgCl_2$, 10mM caesium-EGTA, 10mM Hepes; pH 7.2 (CsOH). Cells were held at a potential of -60 mV. Current–voltage (I–V) relationships were obtained from voltage ramps from -90 to +60 mV, applied over 200 ms. The experiments were performed at room temperature.

Off-line analysis was performed using a CED1401+ interface (www.ced.co.uk), personal computer and Signal software (www.ced.co.uk).

2.11 Biotinylation of Cell Surface

Fresh neutrophils were resuspended in PBS (containing Ca^{2+} and Mg^{2+}) at $5x10^6$/ml and biotin (EZ-Link, Sulfo-NHS-Biotin Reagent) was added to a final concentration of 0.1mg/ml and left for 1 h on ice. Neutrophils were then washed 3 times in PBS

(containing Ca^{2+} and Mg^{2+}) and the cells were lysed as described in whole cell lysis section 2.12

2.12 Whole Cell Lysis Method

Neutrophils (5 x 10^6 cells/ml per sample), were lysed, following stimulation as detailed in figure legends, in lysis buffer containing 100 mM Tris HCl (pH 8.0), 100 mM NaCl , 2 mM EDTA, 1% v/v Nonidet NP-40, 5 mM Na_3VO_4, 50 mM NaF & protease inhibitor cocktail for 30 min at 4°C. Samples were centrifuged for 20 min at 15,000 rpm at 4°C and supernatants were incubated with electrophoresis sample buffer under reducing conditions which contained 0.25M Tris HCl (pH 6.8), sodium dodecylsulphate (8% w/v), β-mercaptoethanol (10% w/v), glycerol (30% v/v) and bromophenol blue (0.02% w/v).

2.13 Immunoprecipitation

Lysates of freshly isolated neutrophils were made as detailed in whole cell lysis method. 1μg-3μg of immunoprecipitation antibody was added to each lysate and left on ice for one hour. A conjugated Sepharose resin was used to pull out the antibody as appropriate and left to spin for 2 h at 4°C. The samples were then centrifuged to pellet the Sepharose and washed four times in whole cell lysis buffer and resuspended to 25μl in whole cell lysis buffer. The samples were then incubated with reducing sample buffer and boiled at 95°C for 5 min. Each sample was loaded onto a 7.5% SDS-polyacrylamide gel and run and blotted using the western blotting protocol.

2.14 Triton X-100 Fractions

Neutrophils (5 x 10^6 cells/ml per sample) were lysed, following stimulation as detailed in figure legends, in 200µl of 2X cytoskeleton stablisation (CSK) buffer (50mM PIPES, 8M glycerol, 2mM $MgCl_2$, 0.4% Triton X-100, 4mM EGTA, 4mM EDTA) + 500µl whole cell lysis buffer per 500µl of 2X CSK used and lysed for 10 min at 4°C. The samples were centrifuged at 14,000g for 10 min at 4°C. Supernatants were removed and these samples were called the Triton X-100 soluble fractions. The pellets were washed once for 3 min in 1X CSK buffer and then resuspended to 200µl in Radioimmunoprecipitation (RIPA) buffer (25mM Tris-HCL, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) and left to lyse for 30 min at 4°C to obtain the Triton X-100 insoluble fraction. Both the soluble and insoluble Triton X-100 fractions were resuspended in reducing sample buffer and boiled at 95°C for 5 min prior to analysis by SDS-PAGE and western blotting as described in the relevant sections.

2.15 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins by running samples through an acrylamide gel. There are two gels, the running gel and the stacking gel. The stacking gel is of very low acrylamide concentration and is used to form the wells into which the protein is loaded. The low acrylamide concentration also allows most proteins to be concentrated at the dye front, so that dilute protein samples can be compared to concentrated samples on the same gel. The higher the acrylamide concentration in the running gel the slower the proteins migrate through the gel. On lower percentage gels proteins go faster, therefore the percentage of the gel was chosen so that the protein of interest would be found in the running gel. After cell lysis samples were loaded onto SDS-PAGE gels which were made up of the following solutions:

Running Gel Solution – 1 running gel (7.5mls)

	7.5%	10.0%
Acrylamide/Bisacrylamide Mix	1.88mls	2.5mls
Separating Buffer (1.5M Tris pH8.8)	1.88mls	1.88mls
H_2O	3.71mls	3.09mls
10% SDS	10µl	10µl
10% Ammonium Persulfate (APS)	30µl	30µl
Temed	6µl	6µl

Stacking Gel Solution – 1 stacking gel (2.5mls)

Acrylamide/Bisacrylamide Mix	375µl
Stacking Buffer (0.5M Tris pH6.8)	625µl
H_2O	1425µl
10% SDS	50µl
10% APS	12.5µl
Temed	2.5µl

2.16 Western Blotting

Cell lysate samples were loaded onto a SDS–polyacrylamide gel, the samples were then run at 80V through the stacking gel and 120V through the running gel. Following electrophoresis, the gels were electrophoretically transferred to nitrocellulose by passing a current of 80V through the gel to the membrane in transfer buffer (25mM Tris, 0.2M glycine pH8.3, 20% methanol) for 1 h. The membranes were then blocked for 1 h in 5% w/v dried milk diluted in Tris-buffered saline pH7.6 containing 0.1% v/v Tween 20 (TBST) and probed with primary antibody overnight in TBST (see figure legends). After washing four times with 1X TBST, blots were incubated with goat anti-mouse immunoglobulin HRP conjugate (1:2500) or with goat anti-rabbit immunoglobulin HRP conjugate (1:2500) antibodies diluted in TBST for 1 h at room temperature. After four times washing in TBST the membranes were incubated with ECL reagent, placed under

BioMax MS-1 X-ray sensitive film, and processed through an X-ray developer (www.xograph.com).

2.17 RNA Isolation and RT-PCR

Freshly isolated neutrophils (25 x 10^7/sample) were pelleted and resuspended in 1ml Trizol and incubated for 5 min at room temperature. 0.2ml of chloroform per 1ml of trizol was added to lyse the cells and the tubes were shaken vigorously by hand for 15 s, followed by an incubation at RT for 3 min. The tubes were then centrifuged at 12000g for 15 min at 4°C. The supernatants settled out into an upper RNA rich phase, an interphase of protein and a lower phase of DNA and protein. The upper phase was removed and collected into fresh tubes and 0.25ml of isopropanol was added to each tube and the RNA incubated on ice for 10 min. After centrifugation at 12000g for 10 min at 4°C, a pellet formed which was washed once with 70% ethanol and then centrifuged at 7500g for 5 min at 4°C. The ethanol was then removed and the pellet air dried for 10 min, after which it was dissolved in 20µl of RNase free water and incubated at 55°C for 10 min.

RNA was then DNAse treated to remove any contaminating DNA using a DNAse kit (www.ambion.com). 0.1 volume (of the RNA) of 10x DNAse1 buffer and 1µl of DNAse 1 enzyme was added to the RNA and mixed gently and incubated at 37°C for 30 min. 5µl DNAse inactivation buffer beads were then added to each tube of RNA and incubated at 37°C for 2 min and centrifuged at 10000g for 1 min to pellet the inactivation buffer beads. The supernatant containing the RNA was then transferred to fresh tubes and stored at -80°C.

The isolated RNA was then reverse transcribed to generate cDNA. The RNA was first assessed for purity and yield by measuring absorbance at 260nm and 280nm. An absorbance reading of 1 unit at 260nm corresponds to 40µg/ml RNA in RNase free

water. The concentration of RNA (μg/ml) = 40 x A260. The ratio of the readings at 260nm and 280nm (A260/A280) provides an estimate of the purity of the RNA.

1μl of oligo DT and 2μg of RNA was made to a final volume of 12μl by the addition of dH_2O and then incubated at 70°C for 10 min. 4μl of 1st strand buffer, 2μl of 0.1M DTT, 0.5μl of 20mM dNTP and 0.5μl of RNAse inhibitor mix was then added and incubated at 42°C for 2 min to equilibrate the mix, after which 1μl of superscript enzyme was added and the mix incubated at 42°C for 50 min and then at 70°C for 15 min. The cDNA was then stored at -20°C until required.

2.18 PCR

PCR reactions were carried out using the following reaction mix for 25μl sample: 19μl RNase free water, 2.5μl of 10x reaction buffer, 0.5μl of 10mM dNTP, 0.75μl of 50mM $MgCl_2$, 0.5μl of 20pmol/μl forward and reverse primer, 1μl of cDNA and 0.25μl *Taq* polymerase. Each time a PCR reaction was run, a positive control reaction using GAPDH primers and a negative control reaction without template cDNA was run, to confirm any product was derived from the cDNA.

Two pairs of specific primers each were used to detect TRPC1, 3, 4 & 6 and one primer pair was used for TRPC2, 5 & 7, primers were synthesised by MWG Biotech (www.mwg-biotech.com). The sequence of the primer pairs used along with the predicted size of their expected amplicons are as follows:

TRPC1:	Forward 5'-ATGTATACAACCAGCTCTATCTTG-3' &
	Reverse 5'-AGTCTTTGGTGAGGGAATGATG-3' (525bp);
TRPC2:	Forward 5'-TCTGGACCATGTTCGGTATG-3' &
	Reverse 5'-GCTACCTCGCTTTGCAGTC-3' (565bp)
TRPC3:	Forward 5'-CTGCAAATGAGAGCTTTGGC-3' &
	Reverse 5'-AACTTCCATTCTACATCACTGTC-3' (388bp).

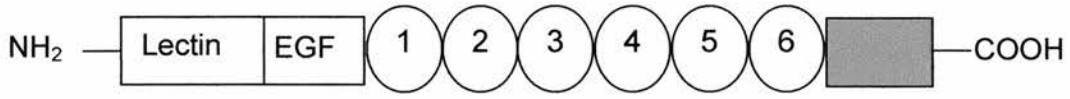
TRPC4:	Forward 5'-ATTCATATACTGCCTTGTGTTG-3' &
	Reverse 5'-GGTCAGCAATCAGTTGGTAAG-3' (329bp).
TRPC5:	Forward 5'-ACTTCTATTATGAAACCAGAGC-3'
	Reverse 5'-GCATGATCGGCAATAAGCTG-3' (289bp).
TRPC6:	Forward 5'-AAGACATCTTCAAGTTCATGGTC-3'
	Reverse 5'-TCAGCGTCATCCTAATTTCCC-3' (322bp).
TRPC7:	Foward 5'-GGAGGATGCAGATGTGGAAT-3'
	Reverse 5'-CACCCTCAGGTGGTCTTTGT-3' (529bp)
STIM1:	Forward 5'-GCGGGAGGGTACTGAG-3'
	Reverse 5' –TCCATGTCATCCACGTCGTCA-3' (500bp)
CRACM1	Forward 5'-GCCAGAGTTACTAGGTG-3'
	Reverse 5'-CGATAAAGATCAGGCCGAAG-3' (585bp)
GAPDH:	Forward 5'-TGCCTCCTGCACCACCAAGTG-3'
	Reverse 5'-AATGCCAGCCCCAGCGTCAAAG-3' (450bp)

The PCR conditions were: 95°C for 10 min, followed by 35 cycles, each consisting of denaturation at 95°C for 1.5 min, annealing at 63°C for 2 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. After PCR amplification, the reaction mixtures were applied to 1% (w/v) agarose gel for electrophoresis and DNA fragments were detected by ethidium bromide staining.

2.19 Expression of E-selectin

Recombinant fusion proteins of E-selectin were obtained using a baculovirus expression construct kindly provided by Dr Mike Bird, GlaxoSmithKline, Stevenage, U.K. Recombinant human E-selectin, lacking the last two consensus repeats, was produced in a baculovirus insect cell expression system as a C-terminal chimera with two protein A domains in tandem (Figure 2.19.1). High five cells (BT1-TN-5B1-4 cell line, Invitrogen) were used to express recombinant E-selectin.

E-selectin



Recombinant E-selectin

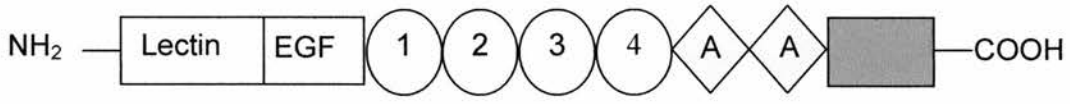


Figure 2.19.1 Recombinant E-selectin Structure

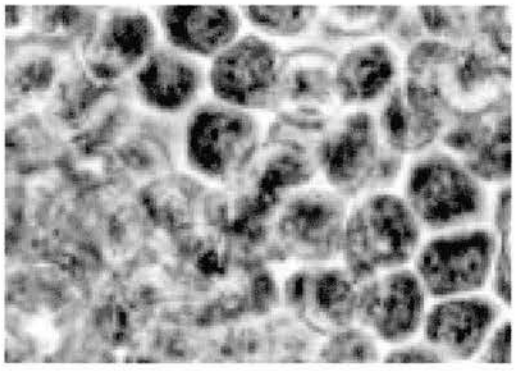
The extracellular portion of E-selectin contains an amino terminal domain homologous to C-type lectin domain and an adjacent epidermal growth factor like domain (EGF). These are followed by six consensus repeat domains (CRD) (numbered circles) and a transmembrane sequence (grey recentangle) and a short cytoplasmic sequence is at the carboxyl terminus. Recombinant E-selectin is comprised of the N-terminal C-type lectin domain, an epidermal growth factor domain and four of the CRDs with two protein A domains (grey diamonds) inserted in tandem at the C- terminal.

High five cells were cultured in Express Five serum-free media supplemented with L-glutamine and penicillin/streptomycin. High Five cells (9×10^6/75cm^2 flask) were seeded into cell culture flasks and left to adhere for 20 min. After attachment of the cells, the medium was removed and the cells were infected with recombinant virus at 2 PFU/cell. Three hours later, the medium was replaced with fresh medium. After 72 h incubation at 27°C, the culture supernatant was collected and stored at 4°C for further purification (Figure 2.19.2).

2.20 Purification of E-selectin

Recombinant proteins were then purified from High Five Insect cell culture supernatants using IgG affinity column chromatography utilising the protein A domains in the recombinant protein. A column (0.7cm x 10cm) containing IgG-agarose was equilibrated with 5 column volumes of 0.1M phosphate buffer pH 8.0. The supernatant from infected High Five cells was applied to the affinity column and the column was then washed with 2 column volumes of 0.1M phosphate buffer before elution with 100mM glycine in 500µl fractions. Fractions were analyzed by SDS-PAGE electrophoresis for protein content (Figure 2.20.1). The eluted proteins were then dialyzed against PBS (Ca^{2+} and Mg^{2+} free) overnight. A typical yield was 1.5-2.0mg of protein per 250ml supernatant.

A



B

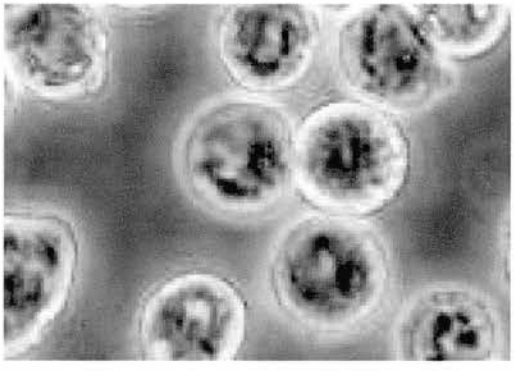


Figure 2.19.2 Expression of E-selectin

Uninfected High Five cells (A), these cells continue to divide and form a confluent monolayer. High Five cells infected with E-selectin baculovirus, after 72 h incubation (B), these cells stop dividing and enlarge and produce E-selectin into the supernatant.

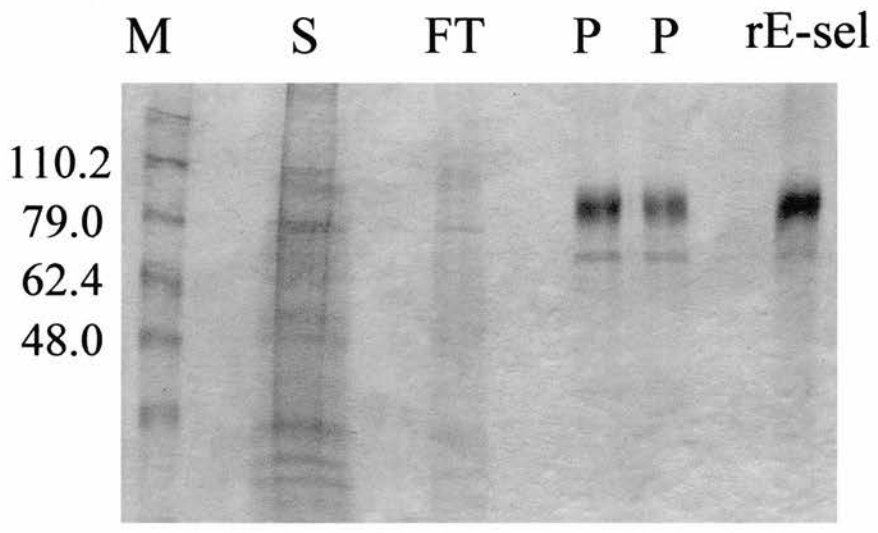


Figure 2.20.1 Purification of E-Selectin

Coomassie blue gel of the different stages of the E-selectin purification process. Soluble E-selectin is approximately 70 KDa in size and recombinant E-selectin (rE-sel) purchased from R&D Systems was used as a positive control. S= E-selectin transfected High Five cell culture supernatant, FT= Flow Through and P = Purified soluble E-selectin.

2.21 Amplification of Virus Stocks

High Five cells were infected at cell concentration of $1x10^6$ cell/ml at a MOI of between 0.01-0.1 PFU/cell and incubated at 27°C with constant agitation between 90-110rpm. During infection 5% (v/v) HI-FCS was added to the culture. Cells were counted the following day and if necessary were diluted to maintain a cell concentration of $2x10^6$ cells/ml. The cell concentration was monitored and maintained at $2x10^6$ until it was observed that cell growth had stopped. The culture was then left for a further 3 days. The virus was harvested from cell culture supernatant by centrifugation at 2000 x g for 30 min and the virus was stored at 4°C in the dark.

2.22 Flow Cytometry Software

All flow cytometry data collected was analysed using the Becton Dickinson Cell Quest software package.

2.23 Statistical Analysis

Data was analysed using the GraphPad Prism statistical package. When more than two conditions were compared, data was analysed by repeated ANOVA with post t-test (Tukey-Kramer Multiple Comparisons Test).

Chapter 3: Identification of Store Operated Calcium Entry in neutrophils

3.1 Introduction

Inflammatory processes expose neutrophils to a wide variety of agonists that can attract, prime, or activate neutrophils via heterotrimeric GPCRs. Neutrophil GPCRs activate a variety of signal transduction pathways including increasing cytosolic Ca^{2+} concentration and phosphorylation of proteins for example MAP kinase and PLC (Chen *et al.*, 2005; Selvatici *et al.*, 2006). Increases in $[Ca^{2+}]_i$ have profound effects on neutrophils, including the initiation of cytoskeletal changes, degranulation, presentation of adhesion molecules, and triggering the oxidative burst. The magnitude and duration of $[Ca^{2+}]_i$ signal responses to G protein-coupled chemoattractants are clearly important (Parekh and Penner, 1997), but the mechanisms by which they are regulated are incompletely understood.

Selectins function not only in shear-dependent tethering and rolling of leukocytes but can also transduce signals that can be involved in cell adhesion, transmigration and release of pro-inflammatory cytokines and proteases. Upon ligation and cross linking they specifically activate neutrophil adhesion (Crockett-Torabi, 1998). The response of neutrophils to various inflammatory stimuli is largely determined by their previous exposure to agents such as cytokines (TNFα), lipid mediators (e.g. PAF) or bacterial products (fMLP). At physiological concentrations these agonists elicit cell polarization, recruitment and activation of β_2-integrins and enhance or 'prime' neutrophil responses (e.g. respiratory burst activation) to other secondary agonists.

Selectin-dependent alterations in neutrophil function have been suggested to require co-stimulation with other agents, e.g. PAF (Lorant *et al.*, 1991; Ostrovsky *et al.*, 1998), fMLP (Kuijper *et al.*, 1996) and TNFα (Carlos and Harlan, 1994). The functional

alterations induced by agents that activate neutrophils appear distinct from those caused by "priming" agents. Early studies have suggested that E-selectin and PAF signalling acts cooperatively to induce neutrophil adhesion to the endothelium.

PAF induces Ca^{2+} mobilization

PAF alone is known to induce Ca^{2+} mobilization through the release of Ca^{2+} from intracellular stores which occurs through activation of the G protein-coupled PAF receptor. G-protein activated PLC produces two secondary messengers: DAG and IP_3. IP_3 production results in the activation of IP_3Rs in the ER which causes Ca^{2+} release and the opening of surface membrane channels which leads to Ca^{2+} influx into the cytoplasmic compartment (Figure 3.1A). These rapid events produce a characteristic Ca^{2+} "spike" that rapidly decays due to the action of Ca^{2+} ATPases pumping Ca^{2+} out of the cytosol back into stores and/or out of the cell (Figure 3.1B & C). PAF induces an increase in $[Ca^{2+}]_i$ which occurs primarily through Ca^{2+} release from IP_3 sensitive intracellular stores followed by an influx of extracellular Ca^{2+} via receptor-operated channels expressed in the plasma membrane. Depletion of intracellular Ca^{2+} stores can also induce Ca^{2+} influx across the PM through SOCs.

SOCE

The molecular basis of Ca^{2+} influx is not well understood (Berridge *et al.*, 2000). Through an unknown mechanism, the depletion of internal Ca^{2+} stores activates SOCs present in the PM. Ca^{2+} influx via SOCs (also known as SOCE) causes a sustained increase in $[Ca^{2+}]_i$, which mediates longer term cytosolic calcium signals and provides a means to replenish intracellular stores (Parekh and Penner, 1997).

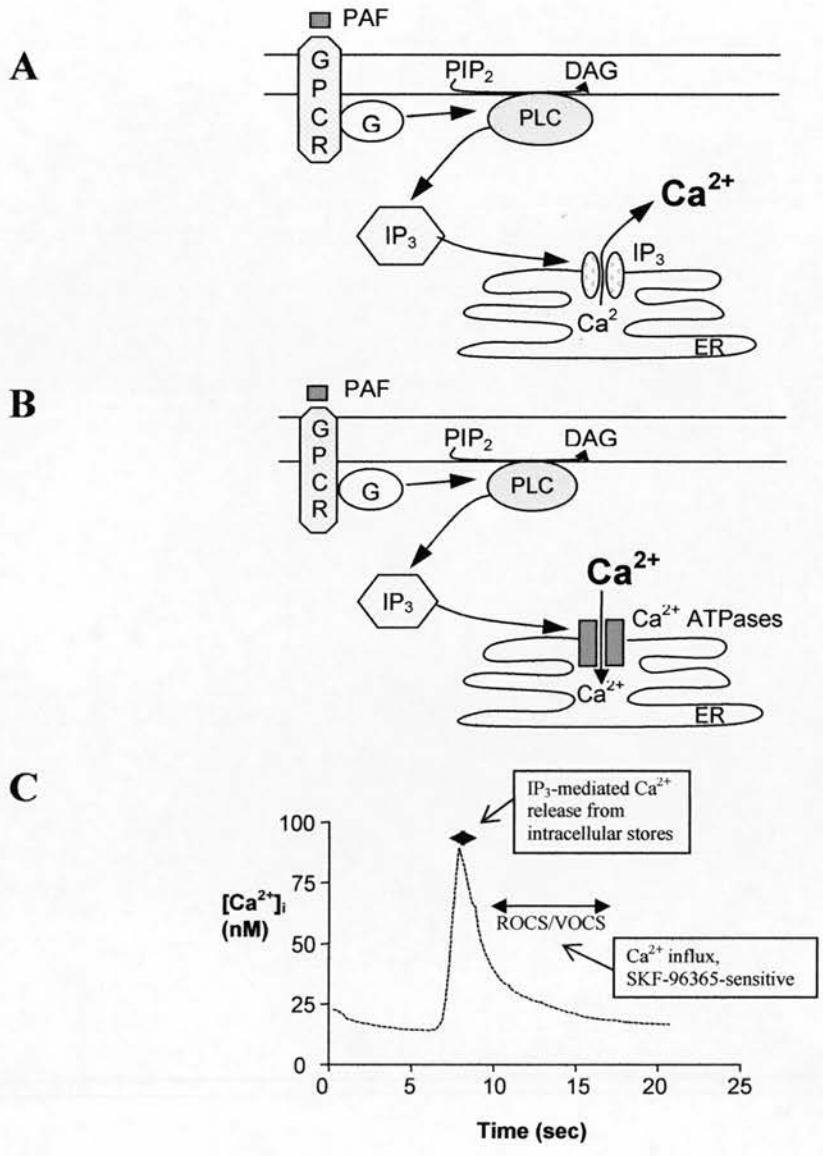


Figure 3.1 PAF induces Ca^{2+} mobilization

(A) Schematic Model of PAF induced Ca^{2+} entry. PAF binds to G-protein coupled receptor (GPCR) resulting in activation of PLC, which cleaves PIP_2 and generates diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). Binding of IP_3 to its receptor (IP_3R) on the endoplasmic reticulum (ER) causes release of Ca^{2+} into the cytoplasm resulting in the initial rapid increase of intracellular Ca^{2+} (B&C). The second phase of the PAF response, shows a rapid decay in $[Ca^{2+}]_i$ as Ca^{2+} is being pumped back into intracellular stores and out of the cell.

Although the molecular identity of SOCs has not been determined, several studies implicate the Ca^{2+} release-activated Ca^{2+} (CRAC) channel as one form of SOC and certain members of the transient receptor potential (TRP) family of cation channels in SOC-channel activity (Parekh and Penner, 1997; Montell, 2001; Lewis, 2007). The two prevailing coupling scenarios to activate SOCs, involve either a diffusible chemical messenger e.g. calcium influx factor (CIF) (Bolotina and Csutora, 2005) or physical interactions between ER and PM (Parekh and Putney, Jr., 2005). However, the nature of the ER-derived signal to activate SOCs remains unresolved.

Recently, a new mechanism of regulation has been suggested following identification of a novel protein termed, stromal interaction molecule (STIM1), which was proposed to act as a Ca^{2+} sensor for the ER and may play a key role in communication with channels in the plasma membrane. This latest model of SOCE regulation may bring us a significant step closer to understanding the intricate complexities involved in SOCE. STIM1 contains a signal sequence and a single transmembrane EF-hand domain and is located in intracellular compartments in the ER at rest. Upon store depletion, STIM1 redistributes from the ER to distinct puncta close to, or within, the plasma membrane (Spassova *et al.*, 2006). Ca^{2+} binding to the EF hand domain of STIM1 within the lumen of the Ca^{2+} store keeps CRAC channels in plasma membrane closed. Mutation of the EF hand motif of STIM1 was found to cause localisation to the plasma membrane, allow basal Ca^{2+} influx to be elevated and caused the CRAC channel current (I_{CRAC}) to operate in a constitutively active, Ca^{2+} store independent manner. (Liou *et al.*, 2005; Spassova *et al.*, 2006). Although the presence or translocation of STIM1 is not sufficient to mediate SOC channel activity (Zhang *et al.*, 2005), it may act as a novel Ca^{2+} sensor in the regulation of SOCE (Liou *et al.*, 2005).

Another regulator of SOCE is CRAC modulator 1 (CRACM1), also known as Orai1 (Feske *et al.*, 2006). Structural analysis of the CRACM1 protein revealed a structure with four proposed transmembrane regions, localisation to the plasma membrane and both N- and C-termini are intracellular (Feske *et al.*, 2006). Overexpression of STIM1

and CRACM1 in HEK293 cells, results in significant amplification of store-operated currents and electrophysiological analysis of channel activity has indicated that these currents are similar to I_{CRAC} (Soboloff *et al.*, 2006). CRACM1 may represent a regulatory protein of a multimeric complex that can influence CRAC channel activity. At present, although the role of these proteins in myeloid cells remains to be defined, altered CRAC channel activity is associated with many immune disorders for example patients with severe combined immunodeficiency (SCID) have a severe defect in CRAC channel activation (Feske *et al.*, 2005), suggesting that they could represent important targets for therapeutic manipulation and intervention.

Recent Studies

Ruchaud-Sparagano *et al.* (2000) demonstrated that soluble E-selectin caused increased adhesion to β_2-integrin ligands (albumin-coated latex beads) treated with PAF. Their study also showed soluble E-selectin and PAF induced β_2-integrin-mediated adhesion required PAF-induced Ca^{2+} mobilization from IP_3-sensitive intracellular stores suggesting that soluble E-selectin might act to alter Ca^{2+} mobilization within neutrophils in response to PAF. Further investigation showed preincubation of neutrophils with soluble E-selectin did not affect the initial increase of $[Ca^{2+}]_i$ in response to PAF (Figure 1C) but caused a subsequent sustained increase in $[Ca^{2+}]_i$ (Ruchaud-Sparagano *et al.*, 2000). However, the mechanism for this prolonged Ca^{2+} response was not defined.

In this chapter experiments will be carried out to investigate the signalling pathways involved in E-selectin's prolongation of PAF induced Ca^{2+} mobilization and data will be shown from experiments aimed at identifying the calcium channel involved. Biochemical evidence will also be provided for molecular cross-talk between these structurally distinct receptor pathways.

3.2 Results

Soluble E-selectin Prolongs PAF-induced Ca^{2+} Mobilization

Incubation of human neutrophils in the presence of soluble E-selectin did not induce Ca^{2+} mobilization (Figure 3.2). However, in agreement with our previous studies (Ruchaud-Sparagano *et al.*, 2000), pre-incubation of neutrophils with soluble E-selectin caused a subsequent sustained increase in $[Ca^{2+}]_i$ in response to PAF without affecting the initial increase of $[Ca^{2+}]_i$ (Figure 3.2). To facilitate comparison of experimental data, we have calculated the area under the Ca^{2+} curve to provide a measure of the Ca^{2+} mobilization observed. Inhibition of soluble E-selectin interaction with neutrophils by ENA2, an anti-E-selectin monoclonal antibody that binds to the lectin domain of E-selectin, inhibited the sustained Ca^{2+} levels without affecting the initial rapid rise in $[Ca^{2+}]_i$ observed in response to PAF treatment (Figure 3.3A&B). These data demonstrate that binding of soluble E-selectin via the lectin domain to a counter receptor present on neutrophils is required for the prolongation of Ca^{2+} mobilization in neutrophils in response to PAF.

The optimum concentration of soluble E-selectin required for the prolongation of Ca^{2+} mobilization in response to PAF was determined by pre-incubating neutrophils with various concentrations of soluble E-selectin before stimulation with PAF. Figure 3.2C shows that 5µg/ml of soluble E-selectin gave the optimal effect. Using a range of pre-incubation times with soluble E-selectin, it was demonstrated that the effects of soluble E-selectin on prolongation of $[Ca^{2+}]_i$ in neutrophils were maximal by 15 min (Figure 3.4A), suggesting that downstream signalling pathways may be required to induce prolonged Ca^{2+} mobilization in response to PAF rather than a rapid physical interaction that exerted direct effects e.g. via conformational changes. Therefore, in all the experiments in this thesis neutrophils were pre-incubated with 5µg/ml soluble E-selectin for 15 min before stimulation with PAF.

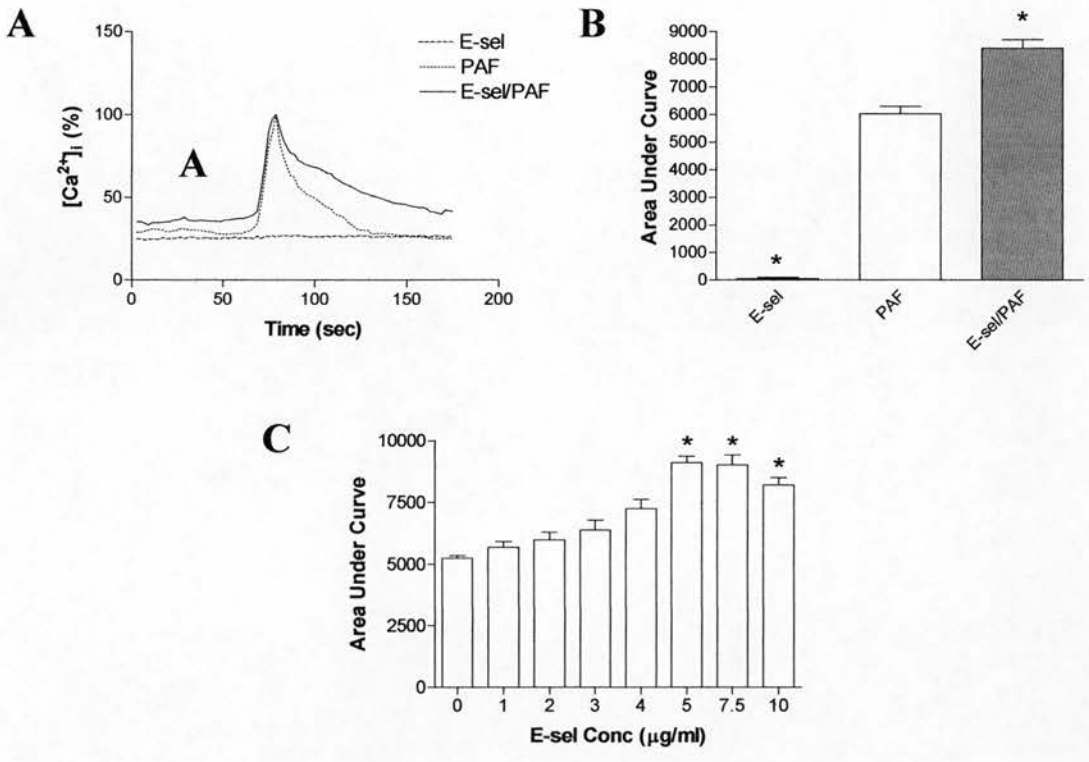


Figure 3.2 Soluble E-selectin prolongs PAF-induced Ca^{2+} mobilization

(A) Freshly isolated neutrophils were loaded with Fura2-AM (2μM) for 30 min at 37°C in Ca^{2+} and Mg^{2+}-free HBSS, then washed and resuspended in HBSS containing Ca^{2+} and Mg^{2+} and then pre-incubated with or without soluble E-selectin (E-sel, 5μg/ml) for 15 min at 37°C as indicated. Neutrophils were stimulated with PAF (100nM) after recording "baseline" Ca^{2+} levels for 60 s. Data is shown as a representative trace of ten separate experiments showing similar results and is expressed as % of peak $[Ca^{2+}]_i$ following PAF stimulation (typically from 50nM control levels to 2.5μM following stimulation). (B) Traces from A have been integrated to calculate area under each curve using GraphPad Prism software (www.graphpad.com) in order to compare the effects of various inhibitors. * Statistically different ($P<0.5$) from PAF treated controls. (C) Freshly isolated neutrophils were loaded with Fura2-AM and preincubated with various concentrations of soluble E-selectin for 5 min. The cells were then stimulated with PAF. * Statistically different ($P<0.5$) from untreated controls.

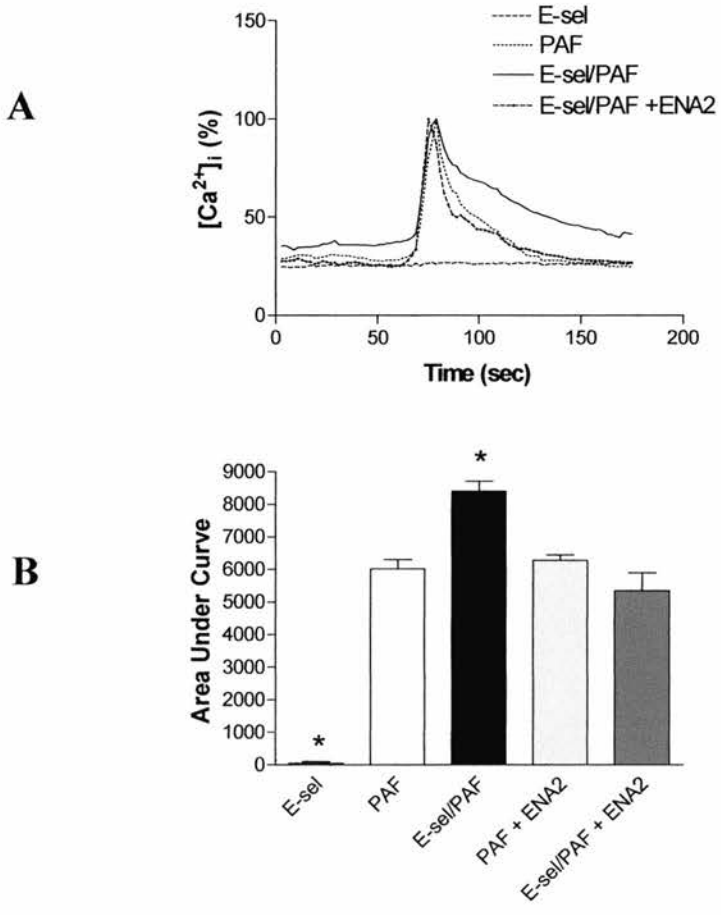


Figure 3.3: Lectin domain of E-selectin required for prolonged Ca^{2+} mobilization

(A) For blockade of CD62E, ENA2 (1:50) and soluble E-selectin were preincubated for 1 h. Freshly isolated neutrophils were loaded with Fura-2-AM (2μM) and preincubated with the E-selectin/ENA2 mixture for 15 min prior to stimulation with 100nM PAF. (B) Bar graph representing area under the curves of the graph in panel A plus other control conditions not included in graph A, calculated using GraphPad Prism software. Data shown are expressed as mean ± SEM from 3 separate experiments that were performed. * Statistically different ($P<0.05$) from PAF-treated control. Data shown are expressed as mean area under the curve ± SEM from 3 independent experiments. * Statistically different ($P<0.05$) from untreated controls.

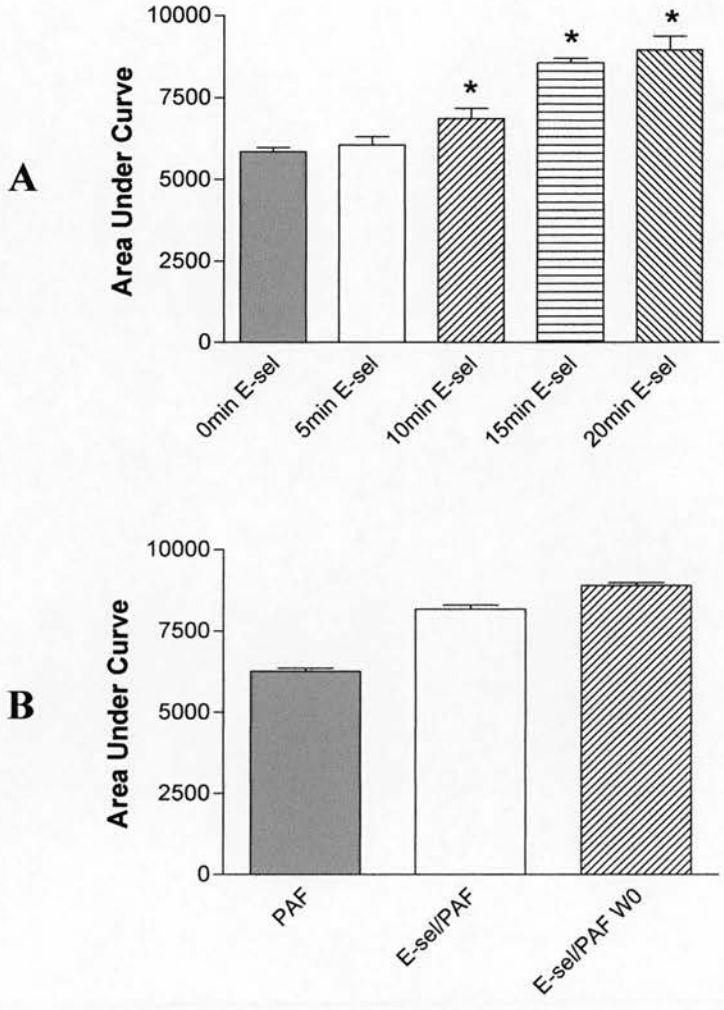


Figure 3.4: Effect of soluble E-selectin is time-dependent

(A) Freshly isolated neutrophils were loaded with Fura2-AM (2μM) as for Figure 1 and pre-incubated with E-selectin (5ug/ml) for various times as indicated. The cells were then stimulated with 100nM PAF after 60 s of recording. Data from three separate experiments that were performed are expressed as mean area under the curve ± sem. * Statistically different ($P<0.05$) from PAF-treated controls. (B) Freshly isolated neutrophils were pre-incubated with soluble E-selectin and samples were either washed to remove E-selectin (WO) or used after 15 min incubation. Data shown are expressed as mean area under the curve ± sem from three independent experiments.

Furthermore, "wash-out" experiments where soluble E-selectin was removed by washing after the 15 min incubation period clearly indicated that the effects of soluble E-selectin upon prolongation of $[Ca^{2+}]_i$ levels were maintained in the absence of extracellular soluble E-selectin (Figure 3.4B). These data indicate that the effects of soluble E-selectin are unlikely to be attributed to non- specific effects such as sequestration or buffering of extracellular Ca^{2+} or changes in associated molecules that affect Ca^{2+} ion movements. One interpretation of these results would be that soluble E-selectin triggers intracellular signalling pathways to modulate Ca^{2+} entry.

Specificity of Soluble E-selectin Effects

In contrast to the effects of soluble E-selectin, pre-incubation of neutrophils with soluble P-selectin had no effect on $[Ca^{2+}]_i$ response to PAF, suggesting that the effect of soluble E-selectin upon Ca^{2+} mobilization is specific (Figure 3.5B). We next examined the ability of soluble E-selectin to prolong Ca^{2+} mobilization in response to other agonists that act via distinct G-protein coupled receptors. As shown in Figure 3.5, in contrast with the effects of PAF, changes in $[Ca^{2+}]_i$ following stimulation with either LTB_4 or fMLP were not affected by pre-incubation with soluble E-selectin. These data are in agreement with previously published results which showed a similar lack of effect with LTB_4 and fMLP-induced Ca^{2+} mobilization (Ruchaud-Sparagano *et al.*, 2000).

PAF, fMLP and LTB_4 work through distinct G protein coupled receptors and we decided to investigate whether these GPCRs coupled to the same G protein by the use of pertussis toxin. Pretreatment of neutrophils with pertussis toxin, which ADP-ribosylates G_i and G_o, was found to inhibit fMLP- and LTB_4-induced Ca^{2+} mobilization but had no effect on PAF-induced Ca^{2+} responses (Figure 3.6A). These results suggest a role for a pertussis toxin-insensitive G_q protein in mediating the effects of PAF and for a pertussis toxin-sensitive G_o or G_i proteins in mediating the effects of fMLP and LTB_4.

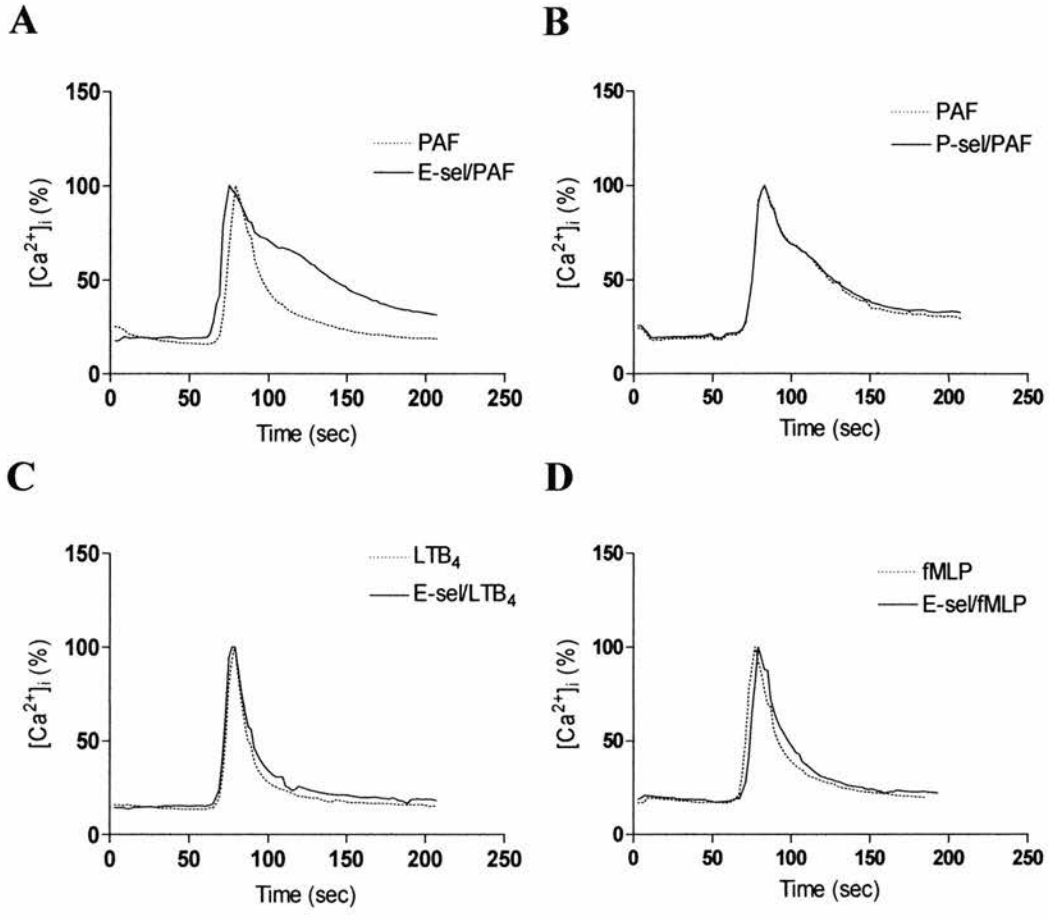


Figure 3.5 Soluble E-selectin effect upon Ca^{2+} mobilization is specific to PAF Freshly isolated neutrophils were loaded with Fura-2-AM (2 μM) and preincubated with or without E-selectin (5μg/ml) or P-selectin (5μg/ml) for 15 min before measuring $[Ca^{2+}]_i$. 60 s after recording, the cells were stimulated with PAF (A&B), LTB_4 (C), or fMLP (D), all used at 100nM. Data are representative of three separate experiments.

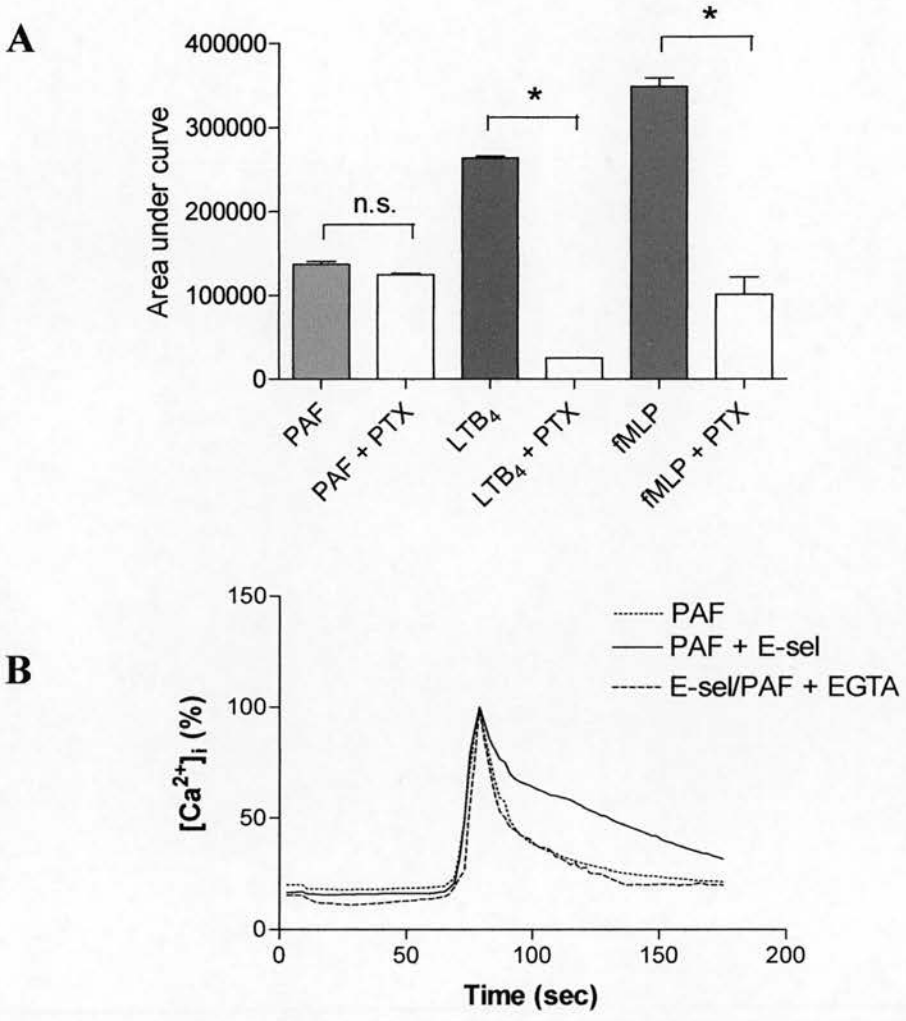


Figure 3.6 Soluble E-selectin specifically modulates Gq agonist induced mobilization

(A) Freshly isolated neutrophils were incubated with Pertussis toxin (2μg/ml) for 1 h, washed and then loaded with Fura2-AM (2μM) for 30 min at 37°C prior to stimulation with 100nM PAF. Data shown is expressed as mean area under each curve ± sem from three separate experiments. * Indicates statistically different ($P<0.05$) from PAF/LTB_4 or fMLP-treated controls. (B) EGTA (1.25mM) was added 10 min before stimulation with 100nM PAF. A representative Ca^{2+} trace from three separate experiments that were performed.

E-selectin permits PAF-induced Ca^{2+} Influx

In a series of experiments in which extracellular Ca^{2+} was chelated by EGTA the E-selectin-mediated prolongation of $[Ca^{2+}]_i$ was found to be sensitive to blockade whereas PAF-induced Ca^{2+} release from stores was unaffected, indicating that soluble E-selectin affected Ca^{2+} influx rather than release from an intracellular store (Figure 3.6B). PAF-induced Ca^{2+} mobilization was dependent on activation of phospholipase C and release of IP_3 as this response was sensitive to complete inhibition by U73122, a specific phospholipase C inhibitor and 2-APB, a specific inhibitor of IP_3-induced Ca^{2+} release (Figure 3.7). Our previous studies also demonstrated that the initial PAF-induced $[Ca^{2+}]_i$ spike was abolished by TMB-8, which blocks Ca^{2+} release from intracellular stores and SKF 96365, a receptor-operated channel inhibitor blocked the PAF-induced extracellular Ca^{2+} influx (see figure 3.1B). However, Ca^{2+} mobilization in the presence of soluble E-selectin was relatively insensitive to SKF 96365, suggesting that soluble E-selectin-induced Ca^{2+} mobilization occurs through distinct ion channels rather than influx of extracellular Ca^{2+} through SKF 96365-sensitive receptor-operated channels.

Store operated or Receptor Operated Ca^{2+} Influx?

SOCs can be activated by the depletion of intracellular Ca^{2+} stores causing a sustained increase in calcium via Ca^{2+} influx. Depletion or emptying of intracellular Ca^{2+} stores and activation of SOCE can be achieved in a number of ways including inhibition of the Ca^{2+} ATPase pumps of the ER. Thapsigargin, a Ca^{2+} ATPase pump blocker, inhibits ER Ca^{2+} uptake directly and as intracellular stores are 'leaky' this results in depletion of the intracellular stores that are involved in agonist-induced Ca^{2+} mobilization. Thus, thapsigargin prevents activation of SOCs and prevents SOCE. To confirm that E-selectin prolonged PAF-induced Ca^{2+} mobilization was SOC mediated rather than ROC we pretreated neutrophils with 2μM thapsigargin.

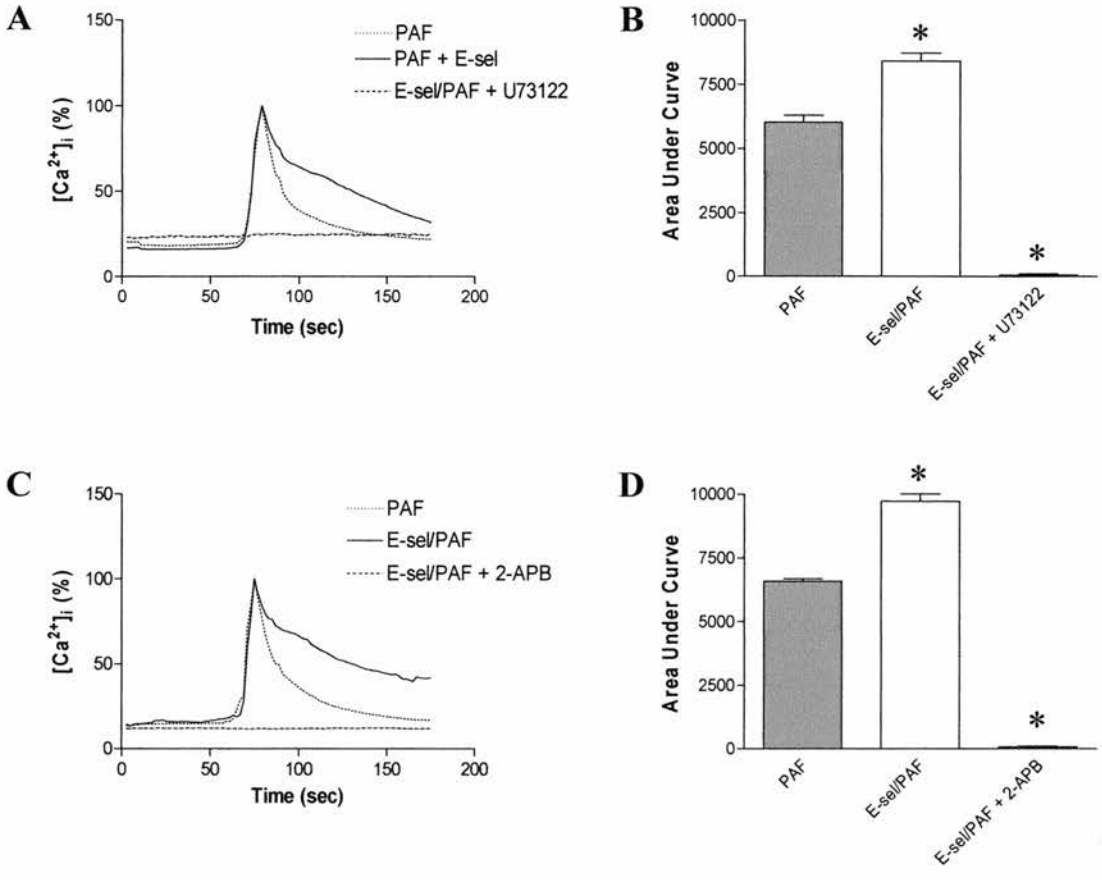


Figure 3.7 Ca^{2+} influx requires PLC activity

(A) U73122 (5μM) was added 5 min before stimulation with 100nM PAF. A representative Ca^{2+} trace from three separate experiments that were performed. (B) Bar graph representing area under the curves of the graph in A, calculated using GraphPad Prism software. Data shown are expressed as mean ± sem from three separate experiments is shown. * Indicates statistically different ($P<0.05$) from PAF treated controls. (C) 2-APB (100μM) was added 5 min before stimulation with 100nM PAF. A representative Ca^{2+} trace from three separate experiments that were preformed. (D) Bar graph representing area under the curves of the graph in C, calculated using GraphPad Prism software. Data shown are expressed as mean ± sem from three separate experiments is shown. * Indicates statistically different ($P<0.05$) from PAF treated controls.

Thapsigargin-treated neutrophils alone did not induce SOCE (data not shown) and Figure 3.8A shows that when thapsigargin-treated neutrophils are stimulated with PAF, it does not induce calcium influx from plasma membrane stores.

These data confirm that PAF induced Ca^{2+} release is from intracellular stores and suggests that store depletion alone or store depletion and a GPCR signal are not sufficient for SOCE to occur. However, we found that pre-incubation of thapsigargin-treated neutrophils with soluble E-selectin before stimulation with PAF significantly attenuated store-operated calcium entry. Figure 3.8B shows that the E-selectin and PAF induced Ca^{2+} increase in neutrophils pre-treated with thapsigargin was sensitive to blockade by chelation of extracellular Ca^{2+} by EGTA confirming that it was Ca^{2+} influx from SOC rather than Ca^{2+} release from an intracellular store. These results suggest that E-selectin 'primes' a special SOC but alone does not allow Ca^{2+} influx to occur as it requires a G protein-derived signal which comes from a G_q receptor such as the PAF receptor.

E-selectin causes Ca^{2+} influx through a TRPC

We therefore sought to further define the molecular mechanism by which soluble E-selectin induced prolonged elevation of $[Ca^{2+}]_i$. Neutrophils treated with ruthenium red, an inhibitor of Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive stores, were found to mobilize Ca^{2+} in response to PAF in the presence of soluble E-selectin in a similar way to those under control conditions (Figure 3.9B). These results further suggest that soluble E-selectin was most likely mediating Ca^{2+} influx through the plasma membrane rather than from an intracellular store. Depletion of intracellular Ca^{2+} stores is known to communicate with and activate SOCs in the plasma membrane, leading to SOCE. The initial elevation of $[Ca^{2+}]_i$ in response to PAF, known to be due to release of Ca^{2+} from IP_3-sensitive stores, was unaffected by MRS1845, a store-operated channel inhibitor (Figure 3.9).

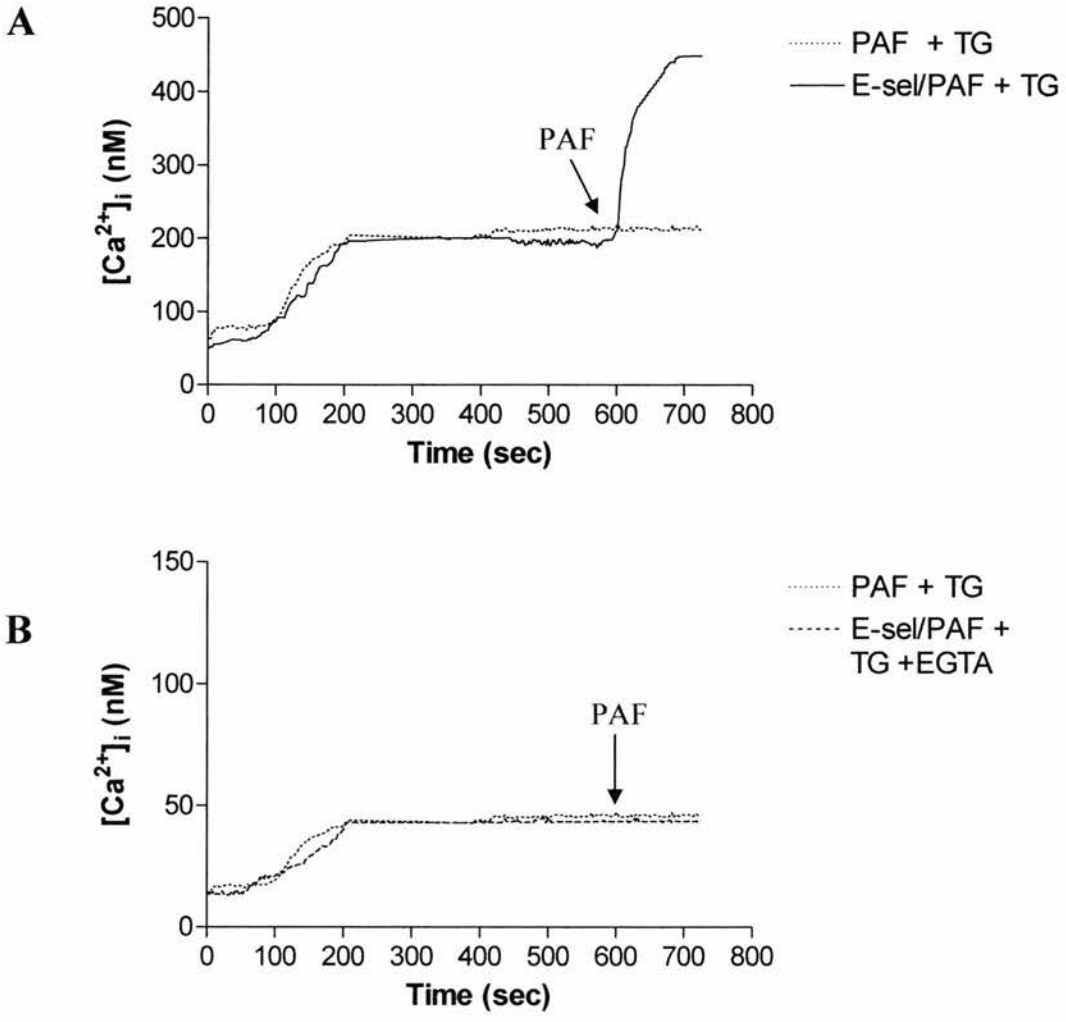


Figure 3.8 Store operated or receptor operated channel?

(A) Thapsigargin (TG) (2μM) was pre-incubated with or without 5μmg/ml E-selectin for 5 min before recording started and cells were stimulated with 100nM PAF at 10 min. The Ca^{2+} trace shown is representative of three separate experiments that were performed with similar results.

(B) Thapsigargin (2μM) was pre-incubated with or without 5μg/ml E-selectin for 5 min before recording started and 1.25mM EGTA was added at time zero before stimulation with 100nM PAF at 10 min. A representative Ca^{2+} trace from three separate experiments that were performed.

Interestingly, the soluble E-selectin-induced sustained $[Ca^{2+}]_i$ increase following stimulation with PAF was inhibited by MRS1845 (Figure 3.9B), indicating that PAF-induced Ca^{2+} store-emptying was required for E-selectin activation of a store-operated channel, an effect we have termed 'permissive' SOCE.

As certain members of the transient receptor potential family of cation channels (TRPC) display properties similar to those of SOCs, experiments were carried out to test whether gadolinium (III) chloride (Gd^{3+}), a specific TRPC inhibitor, would affect the soluble E-selectin-induced $[Ca^{2+}]_i$ response to PAF. As shown in Figure 3.9A, the prolonged rise in $[Ca^{2+}]_i$ observed in the presence of soluble E-soluble was sensitive to Gd^{3+} which would be consistent with a role for TRPC in this response.

TRPC expression in PMN

To determine the profile of TRPC expression in human neutrophils, multiple specific primer pairs were used to screen for the presence of TRPC1-TRPC7 mRNA species in highly purified human neutrophils. The expression profile for members of the TRPC family is illustrated in a representative gel of RT-PCR products shown in Figure 3.10A. PCR products for TRPC6 were found in all neutrophil samples (n=10) whereas TRPC3 was only found in 20% of samples. We did not observe signals for TRPC1, 2, 4, 5 and 7 in any of the preparations, despite positive RT-PCR controls demonstrating that these PCR reaction conditions were optimal. Monocytes were used a positive control for TRPC1, 3, 5 and 6 (Figure 3.10B). To confirm TRPC6 protein expression, we assayed crude membrane preparations from freshly isolated human neutrophils using western blotting techniques. A specific antibody for TRPC6 revealed a strong band in the appropriate 90-100KDa range (Figure 3.11A), which could be blocked by a TRPC6 blocking peptide (Figure 3.11B), confirming the presence of protein and the RT-PCR data. However, we could not detect TRPC3 at a protein level (Figure 3.11B), suggesting TRPC6 is the principal TRPC present in neutrophils.

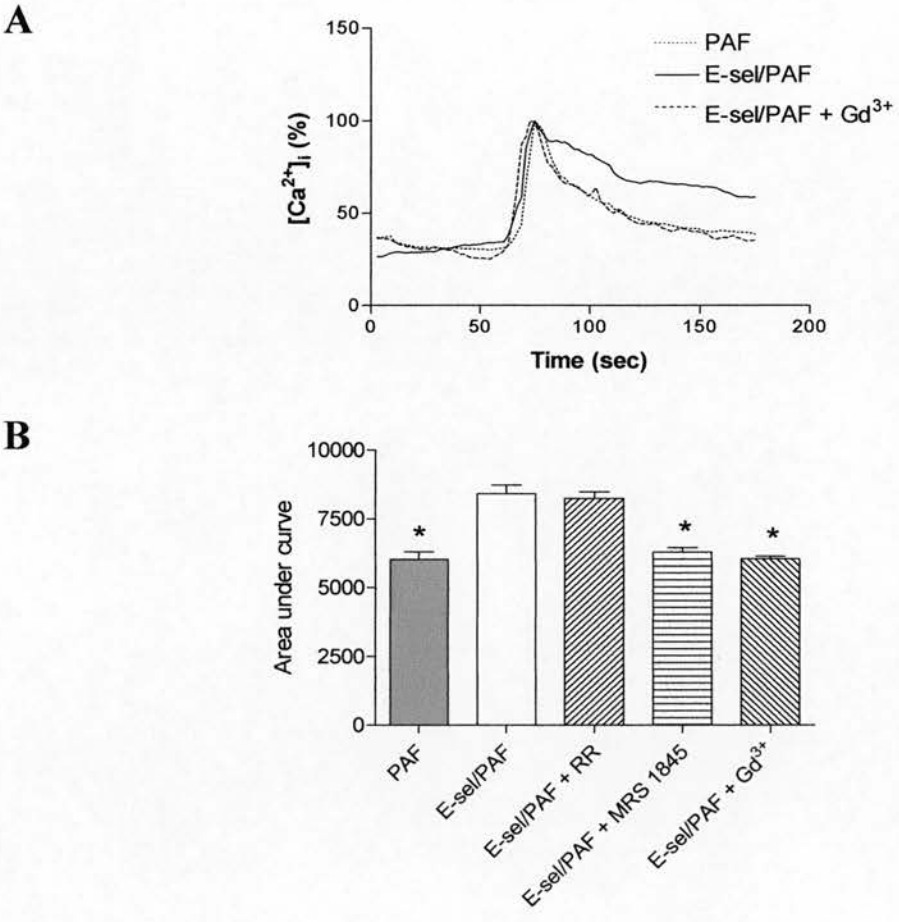


Figure 3.9 TRPCs mediate soluble E-selectin prolongation of PAF-induced Ca^{2+} mobilization

(A) Calcium traces showing the effect of calcium channel inhibitor, Gd^{3+}, on the prolonged $[Ca^{2+}]_i$ elevation induced by soluble E-selectin. $Ga^{3+}Cl_3$ (10μM) was added 3 min before stimulation with 100nM PAF. The calcium trace shown is representative of three separate experiments with similar results. (B) Ruthenium Red (RR, 20nM) or MRS 1845 (2μM) were added 5 min and Gd^{3+} (10μM) was added 3 min before stimulation with 100nM PAF. Bar graph representing area under the curves of graph C plus curves obtained for the other channel inhibitors used in the same experiment, calculated using GraphPad Prism software. Data shown is expressed as mean ± sem from three separate experiments that were performed. * Indicates statistically different ($P<0.05$) from PAF/E-selectin treated controls.

Identification of STIM1 and CRACM1

The precise mechanism of SOCE has remained elusive but identification of STIM1/CRACM1 interactions has recently emerged as the most likely candidate for the missing link between Ca^{2+} store depletion and SOC influx. STIM1 has recently been proposed to be a novel Ca^{2+} sensor involved in the regulation of store-operated Ca^{2+} entry, serving as a calcium sensor that translocates upon store depletion to the plasma membrane to activated SOCs (Baba *et al.*, 2006; Wu *et al.*, 2006). Therefore we looked at expression of the calcium sensors, STIM1 and CRACM1, in human neutrophils. The expression profile for STIM1 and CRACM1 is illustrated in a representative gel of RT-PCR products shown in Figure 3.12. Bands of PCR products of the expected size were consistently obtained for STIM1 and CRACM1 and for the positive control GAPDH in all neutrophil samples (n=5). These results raise the possibility that STIM1 and CRACM1 may also be involved in the SOCE pathway in human neutrophils and have a role in soluble E-selectin prolonged PAF induced Ca^{2+} mobilization.

DAG does not cause Ca^{2+} influx

Recent studies have shown that TRPC channels can be directly activated by increases in intramembrane DAG (Hofmann et al., 1999; Venkatachalam et al., 2003). Hofmann *et al.* (1999) proved that the TRPC subgroup: TRPC3, TRPC6 and TRPC7 can be activated in response to DAG through a mechanism independent of PKC. Therefore, studies investigating the effect of the cell permeable DAG analogue, 1-oleoyl-2-acetly-sn-glycerol (OAG), which directly activates PKC were carried out. Figure 3.13A shows that neutrophils did not mobilise Ca^{2+} upon addition of 100μM OAG, a concentration which several other groups have reported to activate TRPC3, TRPC6 and TRPC7 channels (Hofmann *et al.*, 1999; Beck *et al.*, 2006).

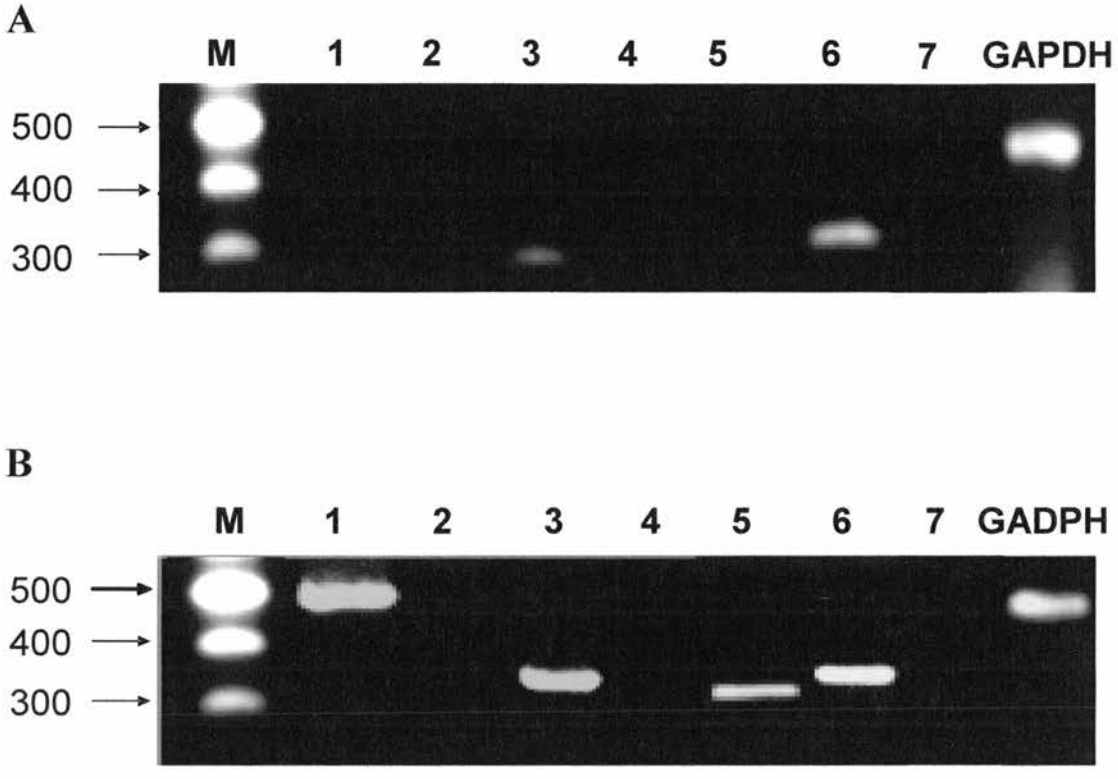


Figure 3.10 TRPC6 expression in human neutrophils

(A) Expression of TRP family members in isolated human neutrophils. Representative results of RT-PCR analysis with mRNA from a single isolation and cDNA preparation of neutrophils with each of the indicated primers are given and GAPDH was used as a positive control. RT-PCR was carried out on ten different cDNA preparations. (B) Expression of TRPC family members in monocytes. Representative results of RT-PCR analysis with mRNA from a single isolation and cDNA preparation of monocytes with each of the indicated primers are given and GAPDH was used as a positive control. Lanes are indicated as M, markers; 1, TRPC1; 2, TRPC2; 3, TRPC3; 4, TRPC4; 5, TRPC5; 6, TRPC6 and 7, TRPC7.

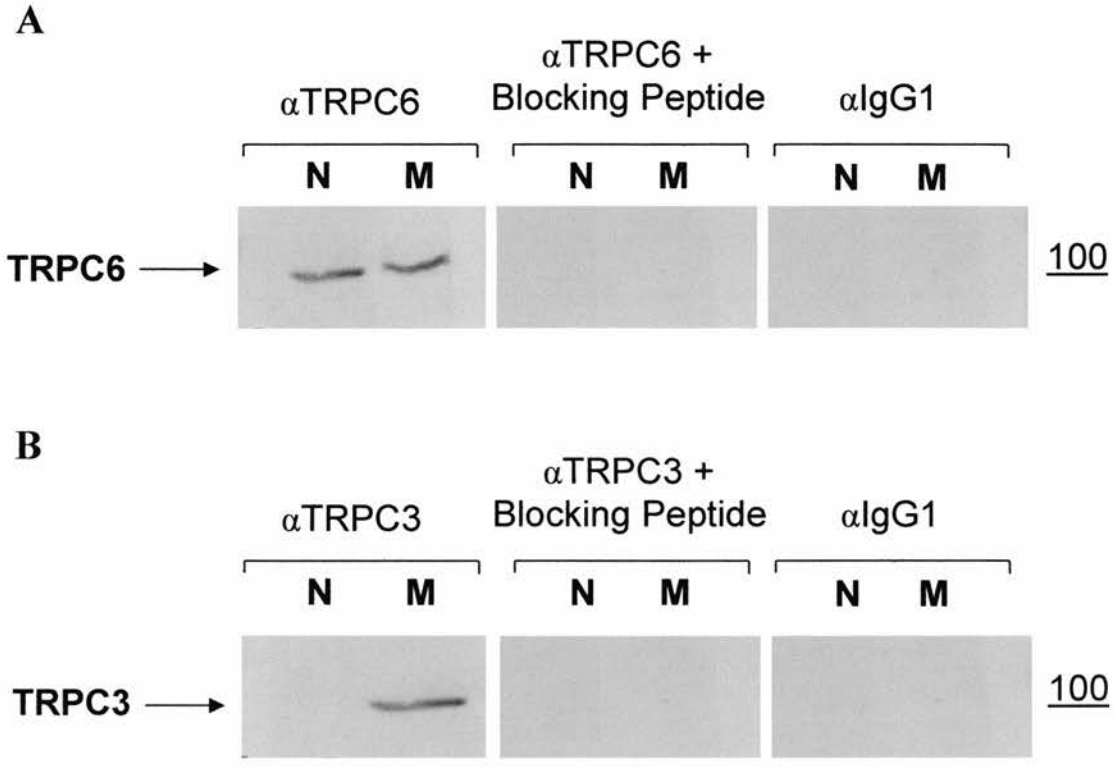


Figure 3.11 TRPC6 expression in human neutrophils

Western blotting of neutrophil (N) or mononuclear cell (M) membrane preparations stained with anti-TRPC6 antibody (1:400) (A) or anti-TRPC3 antibody (1:400) (B) revealed a strong band at the predicted molecular weight (100kDa) as indicated. Specificity was demonstrated by incubation of the TRPC6 antibody with a four-fold excess of the antigenic peptide exhibited no signal. Non-specific binding was assessed using an IgG control stained with rabbit anti-IgG antibody (1:400). Representative immunoblots of three different experiments are shown.

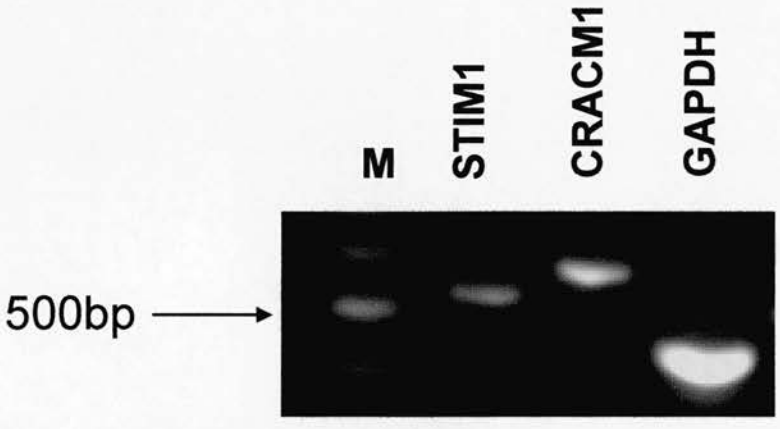


Figure 3.12 Identification of STIM1 & CRACM1 in human neutrophils by RT-PCR

Expression of STIM1 and CRACM1 in isolated human neutrophils. Representative results of RT-PCR analysis with mRNA from a single isolation and cDNA preparation of neutrophils with each of the indicated primers are given and GAPDH was used as a positive control. RT-PCR was carried out on five cDNA preparations and all experiments gave similar results. M = markers.

Priming the channel allows Ca^{2+} influx to be induced by DAG

Although neutrophils did not respond to the addition of OAG alone, prior store depletion using thapsigargin before the addition in OAG resulted in a Ca^{2+} influx. Figure 3.13B shows that the OAG induced Ca^{2+} influx in neutrophils pre-treated with thapsigargin was sensitive to blockade by EGTA-mediated chelation of extracellular Ca^{2+} confirming that it was Ca^{2+} influx from SOCE rather than Ca^{2+} release from an intracellular store. Figure 3.8A shows that when thapsigargin-treated neutrophils are pre-incubated with E-selectin, it did not induce Ca^{2+} influx but when thapsigargin-treated neutrophils are incubated with E-selectin and activated by PAF calcium influx occurs as it requires a G protein-derived signal. The thapsigargin/OAG data may suggest that OAG needs another signalling event to prepare the channel for activation by OAG. Figure 3.14 shows OAG causes a small Ca^{2+} influx in neutrophils pre-incubated with soluble E-selectin. However, OAG did not affect soluble E-selectin-induced modulation of Ca^{2+} influx following stimulation of neutrophils with PAF (Figure 3.14B). These results suggest that the TRPC involved requires to be 'primed' before OAG activation or direct DAG activation independent of PKC, as it requires another signal which comes from soluble E-selectin or store depletion for Ca^{2+} influx to occur.

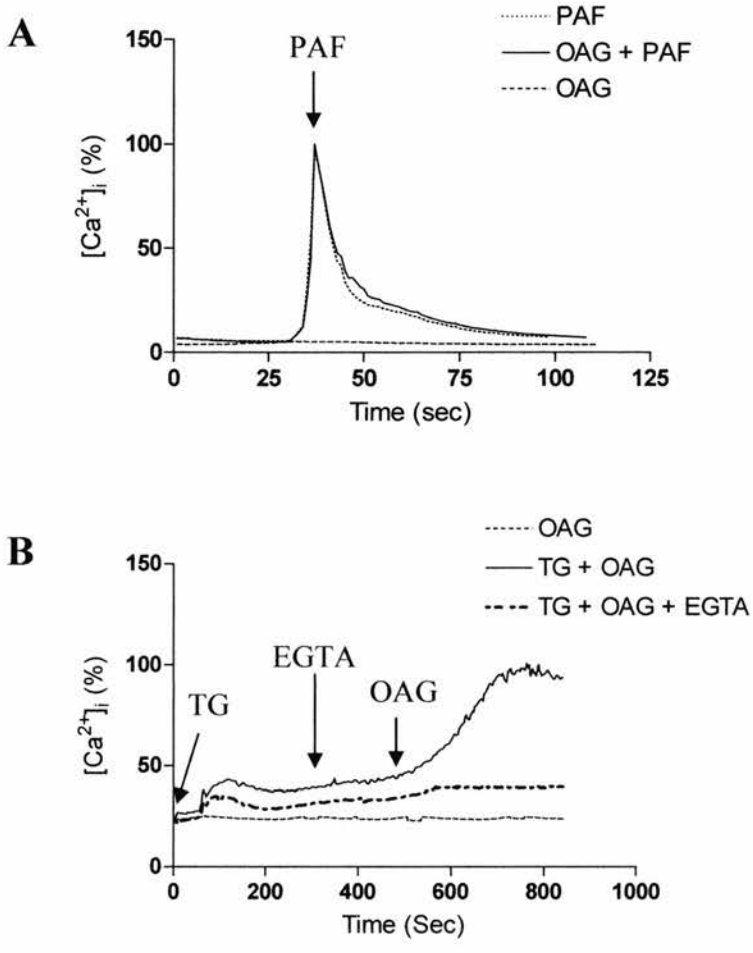


Figure 3.13 DAG alone does not directly activate TRPCs present in human neutrophils

(A) Freshly isolated neutrophils were loaded with Fura-2-AM (2μM) before incubation with 100μM OAG. Before stimulation with PAF, neutrophils were preincubated with 100μM OAG for 15 min. (B) Thapsigargin (TG) (2 μM) was added at 0 min, 100μM OAG was added after 8 min. For EGTA experiments, 1.25mM EGTA was added after 2 min before addition of OAG at 8 min. Graphs are representative Ca^{2+} traces from three separate experiments performed.

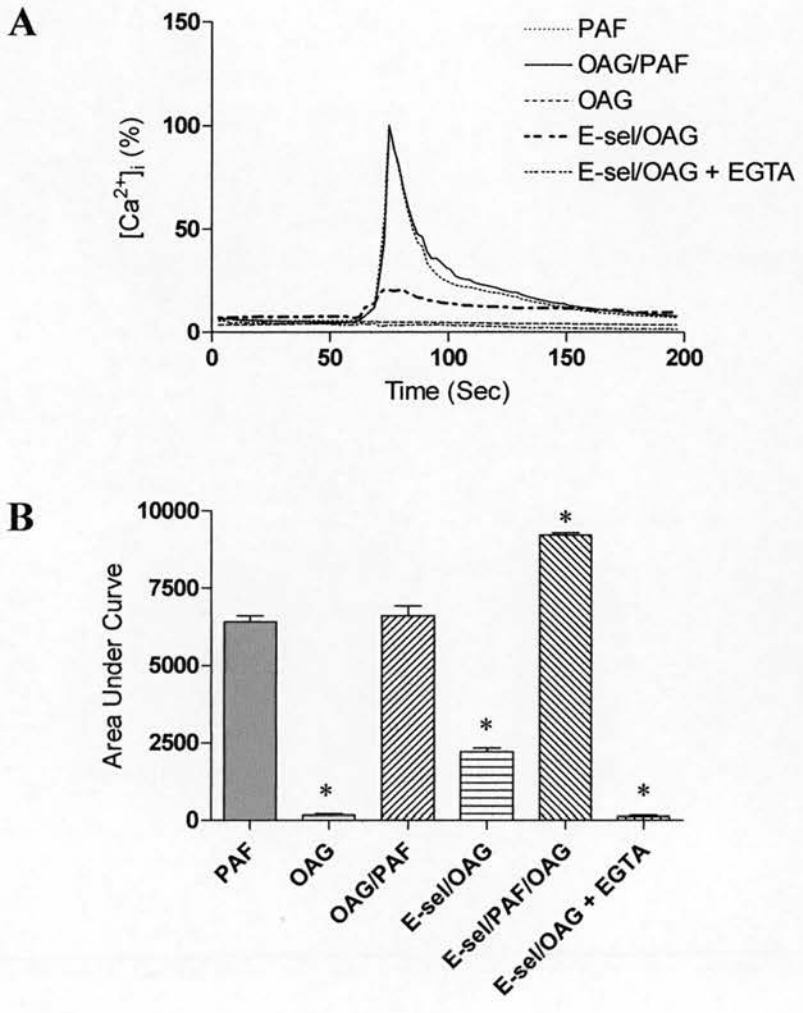


Figure 3.14 E-selectin 'primes' channel for DAG activation

(A) Neutrophils were preincubated with or without soluble E-selectin (5μg/ml) for 15 min before stimulation with 100μM OAG or 100nM PAF. 1.25mM EGTA was added 10 min before stimulation with 100μM OAG. A representative Ca^{2+} trace from three separate experiments that were performed. (B) Bar graph representing area under curves of the graph in A and an E-sel/PAF/OAG treated control, calculated using GraphPad Prism software. Data shown are expressed as mean ± sem from three separate experiments. * Indicates statistically different ($P<0.05$) from PAF treated controls.

Patch Clamp Channel Recordings

As there are few specific TRPC pharmacological inhibitors that are cell permeable, we decided to carry out several whole cell patch clamp experiments to further investigate the role of TRPC channels. Whole-cell patch-clamp is a technique for measuring currents passing across either the whole-cell membrane or through single membrane channels. A small patch of membrane is sealed to the tip of a micropipette and a patch is created. For the whole-cell recording technique this patch is then ruptured thereby allowing electrical continuity with the inside of the cell and thus permitting current flow across the total cell membrane to be measured. This is a very difficult and technically demanding technique and before experiments could be started buffers had to be designed which were suitable for TRPC channel recording and would not activate neutrophils. A surface to which neutrophils would attach to but not adhere to and become activated was then identified. By coating glass cover slips with Sigmacote, this allowed us to attach recording pipettes to non-activated neutrophils and prevented the neutrophils from firmly adhering to the glass cover slips.

In order to determine the identity and biophysical properties of the TRPC channel in human neutrophils which are modulated by soluble E-selectin and PAF whole-cell, patch-clamp recordings were carried out. Figure 3.15B shows membrane currents obtained from a resting neutrophil using the whole-cell recording configuration. The neutrophil was voltage-clamped at -55 mV and then stepped briefly to a range of potentials indicated by the corresponding voltage commands shown in Figure 3.15A. As can be seen from the amplitude of the responses, current flow across the cell membrane is very small suggesting very few channels are open under these conditions. This would be expected as a caesium based internal pipette solution which blocks K^+ channels was used. Figure 3.15C shows the current-voltage relationship for this neutrophil. The slope of the current-voltage relationship obtained by linear regression gives a whole-cell conductance of 0.58nS. The conductance is an indication of the number of channels open in the cell membrane.

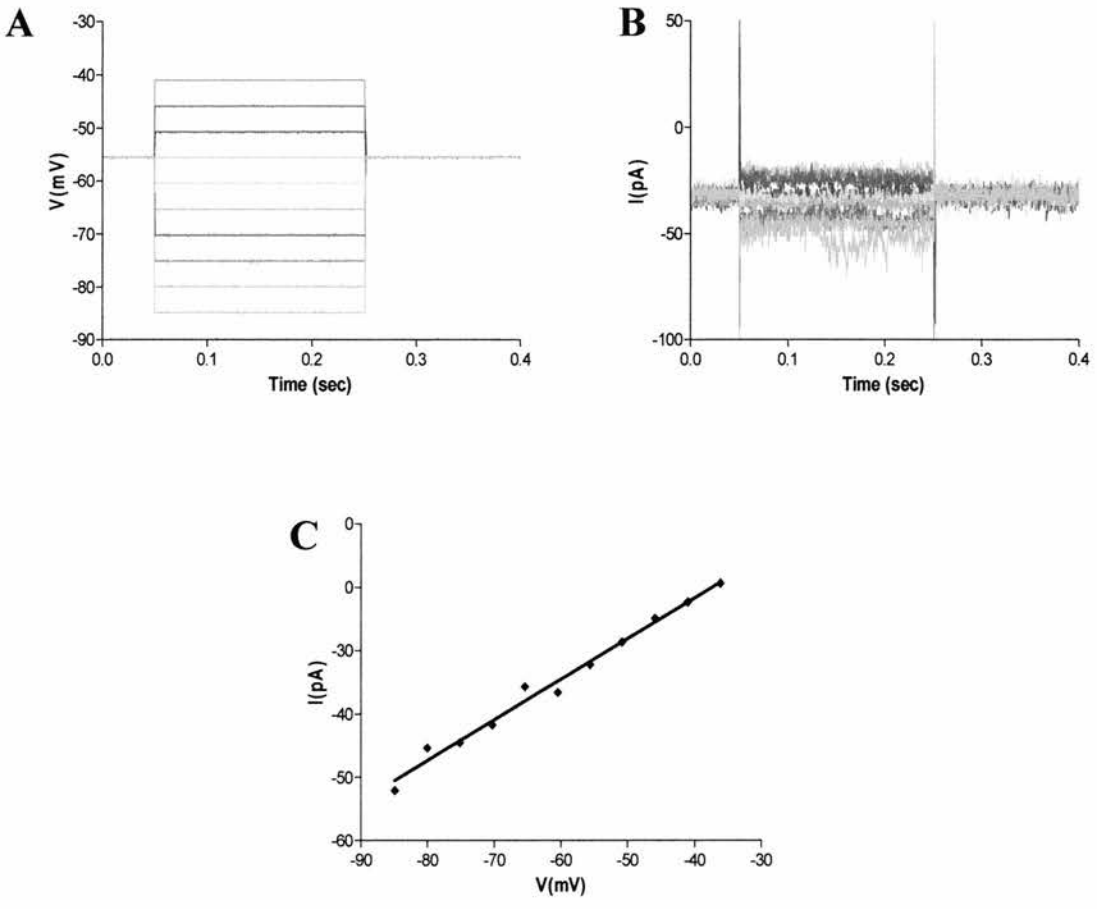


Figure 3.15 Whole-cell patch-clamp recording from a resting neutrophil

(A) Whole-cell current responses to voltage step commands shown in (B) obtained from a neutrophil in normal external solution. The neutrophil was voltage-clamped at -55mV. (C) Current-voltage relationship obtained from the records shown in A and B. The line has been fitted by linear regression and indicates a resting whole-cell conductance of 0.58nS.

This is useful because it allows you to quantify any changes observed as a consequence of drug action, for example an increase in conductance (as indicated by an increase in the slope of the I-V plot) indicates channels opening.

Under the same experimental conditions as described above OAG, a derivative of DAG which is known to activate TRPC channels, was bath-applied to a different neutrophil (Figure 3.16C). Current-voltage relationships were generated before and during OAG (100 μM) application (Figure 3.16C) using a voltage ramp protocol illustrated in Figure 3.16A. OAG caused a large increase in conductance from 1.02nS to 4.53nS indicating the opening of channels in the cell membrane. The control and OAG current-voltage plots intersect at 8mV. This represents the reversal potential for the current modulated by the drug and gives an indication of the ions flowing through the open channels. PAF was also bath-applied to a different neutrophil, under the same experimental conditions (Figure 3.16B). Current-voltage relationships were generated before, during PAF (100nM) and after (washout) of PAF using a voltage ramp protocol illustrated in Figure 3.16B.

PAF caused a significant increase in conductance from 0.47nS to 1.19nS indicating the opening of channels in the cell membrane. The washout current-voltage plot gave a conductance of 0.72nS indicating the PAF response is reversible and is unlikely to be an artefact. The control and PAF current-voltage plots also intersect at 8mV, which represents the reversal potential of the current induced by the drug. Reversal potentials of 8mV after the addition of PAF or OAG suggest a non-selective cation channel has been opened. This could include permeability to Ca^{2+} though further extensive work would be required to confirm that it is Ca^{2+} entering the cell and that this occurs through the TRPC6 channel.

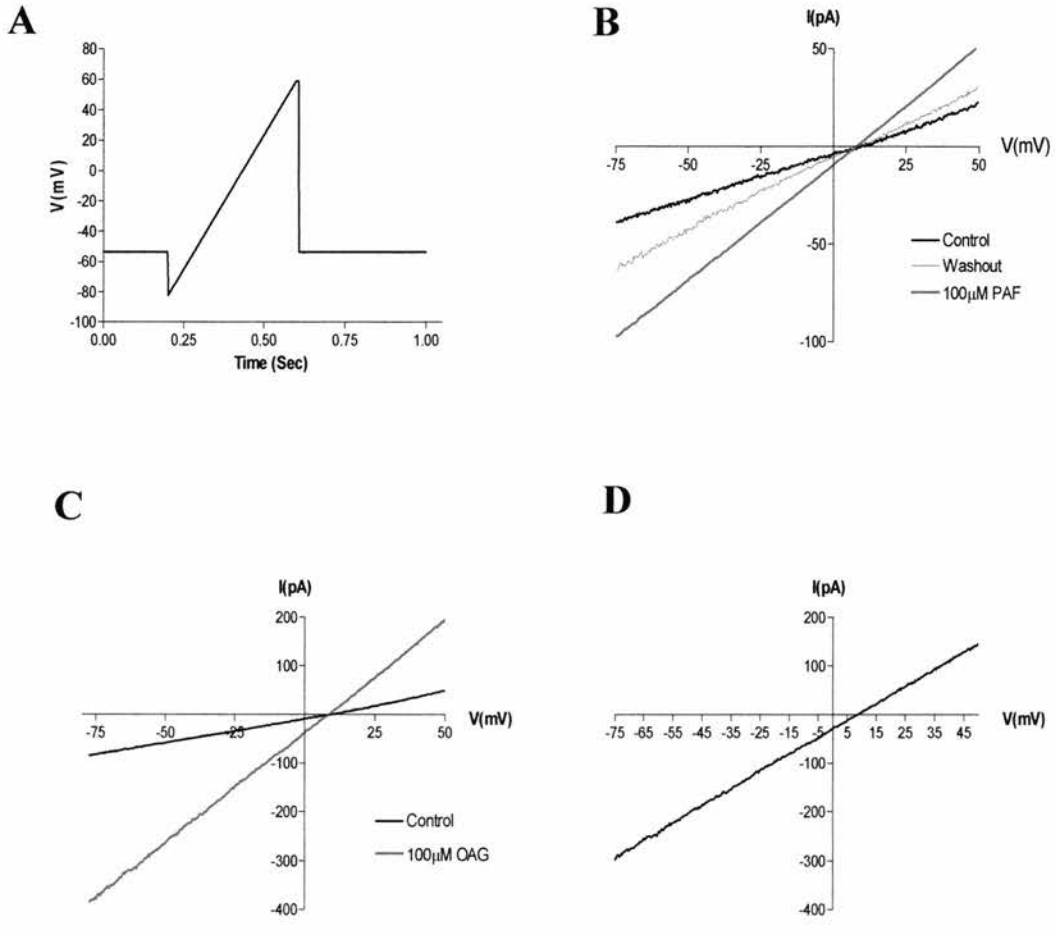


Figure 3.16 Activation of membrane currents by PAF & OAG

Current responses were obtained using the ramped voltage protocol shown in (A). The voltage was ramped from -85 to +60 mV at a rate of 210mV/s. (B) Current-voltage relationships obtained from a resting neutrophil before (Control), during (PAF) and after (Washout) bath-application of PAF (100μM). (C) Current-voltage relationships obtained from a resting neutrophil before (Control), and during (OAG) bath-application of OAG (100μM). (D) Current-voltage relationship showing the OAG sensitive current obtained by subtracting the current-voltage plot obtained in the absence of the OAG from that obtained in presence of OAG. The reversal potential of OAG is 8mV.

3.3 Discussion

The selectin family of receptors are critical for the appropriate recruitment of neutrophils to sites of infection or tissue injury and the initiation and progression of the inflammatory response. Soluble E-selectin acts to promote integrin-mediated neutrophil adhesion, inhibit migration and amplify destructive responses, raising the possibility that elevated levels of soluble E-selectin in patients with inflammatory diseases such as rheumatoid arthritis and being associated with tumour growth may have pro-inflammatory effects (Koch *et al.*, 1993). Importantly, a requirement for the lectin-binding domain of E-selectin implies that a putative E-selectin receptor is most likely involved in mediating these responses. Ligation of selectin receptors has been reported to transduce intracellular signals resulting in altered PAF Ca^{2+} mobilization and β_2 integrin-mediated adhesion (Ruchaud-Sparagano *et al.*, 2000). Here we show prolongation of PAF induced Ca^{2+} mobilization by soluble E-selectin is maintained following 'wash-out' suggesting that soluble E-selectin induces activation of signalling pathways.

In leukocytes, PAF acts through its specific G protein-coupled receptor to induce chemotaxis. Neutrophil activation by PAF has been shown to be pertussis toxin-insensitive implicating G_q, whereas stimulation by LTB_4 and fMLP are pertussis toxin-sensitive, suggesting G_o or G_i involvement in mediating these responses (Lad *et al.*, 1985; Powell *et al.*, 1996; M'Rabet *et al.*, 1999). In neutrophils, β_2 integrin-mediated adhesion to albumin-coated latex beads induced by PAF but not fMLP and LTB_4 was promoted by soluble E-selectin (Ruchaud-Sparagano *et al.*, 2000). Furthermore, we have also shown that soluble E-selectin specifically prolongs elevation of $[Ca^{2+}]_i$ in response to PAF but not fMLP or LTB_4 (Ruchaud-Sparagano *et al.*, 2000). Our data shows that pertussis toxin does not affect PAF-induced Ca^{2+} mobilization but abolishes fMLP and LTB_4-induced Ca^{2+} mobilization. Several other G_q-coupled agonists including Par2 and thrombin were examined to determine whether they could replicate the E-selectin prolonged Ca^{2+} mobilization response in response to PAF. However, no

other G_q-coupled agonist that either was present on neutrophils or produced a large enough Ca^{2+} response to measure the difference between soluble E-selectin treated and untreated neutrophils. Taken together, this would indicate that only signals from a pertussis toxin-insensitive Gq-coupled receptor such as the PAF receptor are able to communicate with E-selectin receptor to affect $[Ca^{2+}]_i$ signalling.

This study has further defined the molecular mechanism of action of soluble E-selectin on modulating Ca^{2+} influx in human neutrophils following stimulation with PAF. Stimulation of neutrophils with PAF caused activation of PLC through a GPCR, release of IP_3 and mobilization of Ca^{2+} from intracellular stores and Ca^{2+} influx through a receptor-operated ion channel, but did not typically cause SOCE. Importantly, soluble E-selectin alone does not cause release of Ca^{2+} from intracellular stores or Ca^{2+} influx, but modulates the Ca^{2+} signals induced by PAF in a novel manner by allowing 'permissive' SOCE to occur, i.e. where E-selectin permits PAF-induced signals to communicate with and activate SOCE. Temporally, PAF-induced release of Ca^{2+} from IP_3-sensitive stores is an essential event that precedes soluble E-selectin-mediated SOCE, suggesting that PAF and E-selectin are not binding to an ion channel and influencing channel regulation or opening directly. In addition, soluble E-selectin is not acting directly on ryanodine-sensitive Ca^{2+} stores or sensitizing these stores to PAF-induced activation signals. Inhibition of E-selectin-mediated prolongation of PAF-induced Ca^{2+} signals was achieved with MRS1845 and Gd^{3+}, indicating that this occurs through a store-operated channel of the TRPC family.

Experiments using thapsigargin (Figure 3.8) confirmed that soluble E-selectin was mediating Ca^{2+} influx through a SOC. They also suggest that soluble E-selectin primes a SOC but alone does not allow influx to occur which confirms our previous data that soluble E-selectin alone does not induce Ca^{2+} release. These experiments also prove that this 'priming' requires a G protein-derived signal from a G_q-coupled receptor via activation of the PAF receptor as thapsigargin-treated neutrophils pre-incubated with soluble E-selectin do not cause Ca^{2+} influx. We also confirmed that a Ca^{2+} influx

occurred when both PAF and E-selectin were present as EGTA inhibited the increase in $[Ca^{2+}]_i$ (Figure 3.8B) demonstrating that this was due to Ca^{2+} influx. The SOC requires signals from both E-selectin and G_q receptor when intracellular Ca^{2+} is increased in order to be activated and allow Ca^{2+} influx.

These observations suggested that soluble E-selectin was acting to promote a novel mechanism of molecular cross-talk that permits PAF-induced signalling pathways to regulate a TRPC in the plasma membrane that is usually inaccessible to activation by PAF, thus permitting SOCE to occur. It is unclear to date whether soluble E-selectin acts to sensitize TRP channels to the signals which mediate SOCE such as calcium influx factor (CIF) and calcium sensors, that recruitment of a regulatory protein to TRP channels occurs as a result of soluble E-selectin-induced signals which permits SOCE or that soluble E-selectin promotes physical interaction between IP_3 channels in the ER with a TRP channel in the plasma membrane. Physical interactions between IP_3 channel and the TRP could be investigated using confocal microscopy or immunoprecipitation techniques. However, to study the role of CIF and calcium sensors on TRP channel activity, experiments would have to be carried out in a relevant transfection expression system.

Proteins homologous to the Drosophila transient receptor potential gene (trp) Ca^{2+} channels that assemble into heterodimeric ion channels are known to be involved in the generation of SOCE. The TRPC family is composed of at least 7 non-selective ion channels (TRPC1-7), encoded for by separate mammalian genes. These family members have been divided into four subgroups (TRPC1; TRPC4 and 5; TRPC3, 6 and 7; and TRPC2) based upon sequence and functional similarities (Clapham *et al.*, 2001). Our RT-PCR studies found that only TRPC3 and TRPC6 mRNA were expressed in PMN (Figure 3.10A), in agreement with Heiner *et al.* (2003). TRPC6 appears to represent the principal TRPC family member present, being detected at both the level of mRNA and protein. It has recently been shown that TRPC6 is externalized to the plasma membrane by the stimulation of a G_q protein coupled receptor (Cayouette *et al.*, 2004), and it has

also been shown that expression of TRPC6 in COS cells increase Ca^{2+} entry in response to stimulation of a G_q-protein-coupled receptor (Boulay *et al.*, 1997). Several preliminary experiments using immunofluorescence microscopy were carried out to image TRPC6 localisation in neutrophils. However, these were unsuccessful as there are only a few commercially available antibodies for TRPC6 all of which only recognise the intracellular epitopes of TRPC6. For detection of translocation of TRPC6 to the plasma membrane upon stimulation, an antibody that would recognise the extracellular domain to see the appearance of TRPC6 at the plasma membrane was required. The TRPC6 antibodies tested are also only recommended for use in western blotting and immunohistochemistry and have not been characterized for use in immunofluorescence analysis. However, once an appropriate antibody is available it would be interesting to investigate whether soluble E-selectin pre-incubation leads to the up-regulation of TRPC6 on the cell surface and establish whether this is sensitive to activation via G_q-coupled receptor-induced signals specifically.

The precise mechanism of SOCE has remained elusive but identification of STIM1/CRACM1 interactions has recently emerged as the most likely candidate for the missing link between Ca^{2+} store depletion and SOC influx. STIM1 and CRACM1 have recently been proposed to act as Ca^{2+} sensors involved in the regulation of store-operated Ca^{2+} entry (Zhang *et al.*, 2005; Roos *et al.*, 2005; Peinelt *et al.*, 2006). Our RT-PCR studies found that both STIM1 and CRACM1 mRNA were expressed in human neutrophils. Preliminary experiments found that STIM1 was detected at a protein level by western blotting (personal communication with Dr Trevor Walker). However, there is currently no commercially available antibody for CRACM1. Upon depletion of Ca^{2+} from IP_3-sensitive ER, STIM1 has been reported to oligomerise at both the ER membrane and in the plasma membrane, potentially close to SOC and form a protein-protein bridge between the two membranes (Draber and Draberova, 2005). This would allow communication to occur through protein/protein interactions and may involve adaptor proteins to recruit other signalling molecules. One study has shown that STIM1 can activate TRPC1, suggesting that STIM1 may be able to activate or regulate the

activity of TRPC channels (Huang *et al.*, 2006). STIM1/CRACM1 may be activating or interacting with TRPC6 following stimulation of neutrophils with soluble E-selectin and PAF but single cell imaging and confocal microscopy would need to be carried out to determine the localisation of STIM1 and CRACM1 in neutrophils following incubation with E-selectin and PAF.

The recent finding that DAG directly activates TRPC3 and TRPC6 may represent an alternative mechanism for activation of these channels via phospholipase C-linked receptors (Hofmann *et al.*, 1999), allowing regulation to occur independently of Ca^{2+} mobilization from intracellular stores. However, figure 3.13 shows that OAG, a DAG analogue, does not directly activate TRPC channels in neutrophils using Fura 2-AM to record $[Ca^{2+}]_i$ measurements. OAG has been found to be a relatively poor activator of TRPC6 (Estacion *et al.*, 2006), as OAG caused only a small current in stably transfected TRPC6 HEK cells compared to other groups who had reported a robust activation of TRPC3 and TRPC6. Channel recordings seem to be dependent on the conditions the cells are recorded in, as OAG has varying degrees of activation in every study carried out. Studies with heterologously overexpressed TRPC6 channels indicate that PLC-dependent agonists activate channels, probably in a DAG-independent manner (Hofmann *et al.*, 1999; Zhang *et al.*, 2006). However, under these experimental conditions, the number of channel units present is presumably much higher than in the case of endogenous channels and whereas the expression of a channel protein subunit probably results in the formation of homomeric channels, the native channels may be constituted by different protein subunits and have different properties (McKay *et al.*, 2000). There are also several other possible explanations as to why OAG does not appear to activate TRPCs in neutrophils; OAG may cause a very small current in neutrophils that is not detectable under our measuring Fura-2AM conditions. Whole cell patch clamp experiments did show that OAG causes a significant influx of divalent ions although further work needs to be carried out to confirm this was Ca^{2+} influx from a TRPC channel. The lack of OAG effect might also be explained as there being a divergence in function of the permeant DAG analogue from the function of the authentic

endogenously generated DAG. OAG may also exert its effect by changing the local lipid environment which allows the channel to open more readily rather than directly activate the channel itself.

Experiments using thapsigargin-treated neutrophils (Figure 3.14) suggest that store depletion primes a SOC for OAG activation but alone does not allow influx to occur. OAG application also resulted in a small Ca^{2+} influx in neutrophils pre-incubated with soluble E-selectin. We confirmed that a Ca^{2+} influx occurred when both E-selectin and thapsigargin were present with OAG, as EGTA inhibited the increase in $[Ca^{2+}]_i$ (Figure 3.14) demonstrating that this was due to Ca^{2+} influx. These results suggest that the TRPC involved requires to be 'primed' before OAG activation or direct DAG activation, as it requires another signal which comes from soluble E-selectin or store depletion for Ca^{2+} influx to occur.

To definitively identify which channels were present in neutrophils and influenced by E-selectin, electrical recording of patch clamp experiments were carried out. It was necessary to spend time developing a method and buffers (in collaboration with Dr Larkman), which were suitable for measuring channel recordings in neutrophils without damaging or affecting the neutrophil's channel activity. We were successful in recording from a resting neutrophil and using TRPC agonists which showed there was detectable channel activity, despite their small size. Due to time constraints and the technical difficulties of patch clamp recording, we were unable to more fully investigate the effects of soluble E-selectin on this channel activity or confirm that a TRPC channel was involved using pharmacological inhibitors. Future experiments include investigation of the identity and biophysical properties of the TRPC channels in human neutrophils which are modulated by E-selectin and PAF and definitive identification of the channel involved in the prolongation of PAF induced Ca^{2+} mobilization by soluble E-selectin. Patch clamp recording is able to distinguish between ion channels as each channel family have their own unique properties, for example each channel has a different single channel conductance, ion permeability and reversal potential. Ion

channels are also activated by different agonists. There are numerous pharmacological tools which can be combined with patch clamp recording to confirm the channel identity. There are several inhibitors of TRPC channels available, however these show varying degrees of specificity. For example, the non-specific cation channel blocker flufenamate can differentiate between the TRPC3, TRPC6 and TRPC7 subfamily members as flufenamate reversibly enhances TRPC6 currents but inhibits both TRPC3 and TRPC7 currents. Therefore, in order to definitively identify the channel involved the combined use of pharmacological blockers with studies examining the conductance and permeability of the channel to different ions. Comparison of the results obtained from these experiments with the properties of expression of a cloned TRPC6 channel. If the profile of the channel obtained in neutrophils matched or was a very close match to the profile of the cloned TRPC6 channel you could definitively say it was a TRPC6 channel involved. This approach would be carried out in parallel with immunohistological studies and/or studies using transgenic animals in which the channel is question has been deleted.

Neutrophils isolated from the bone marrow of $TRPC6^{-/-}$ mice could be used to confirm the role of TRPC6 in E-selectin mediated Ca^{2+} prolongation. $TRPC6^{-/-}$ neutrophils would be pre-incubated with murine E-selectin and we would expect that E-selectin would have no effect on PAF-induced Ca^{2+} mobilization. This technique could also be used to confirm that E-selectin and PAF cause permissive SOCE in neutrophils from mice, in the same way as human neutrophils. Inflammatory neutrophils isolated after migration to an inflammatory site could be used to test if transmigration across soluble E-selectin primes SOCE. Inflammatory neutrophils would be prepared from mouse peritoneal neutrophils which are isolated 4 hours after a peritoneal injection of thioglycollate solution which causes neutrophil activation. However, the use of murine neutrophils may not be a suitable technique as typical yields from bone marrow neutrophil and peritoneal neutrophil (inflammatory neutrophils) preparations are approximately 1-2 million neutrophils, which is equivalent to the amount of neutrophils required for one $[Ca^{2+}]_i$ experiment.

All $[Ca^{2+}]_i$ spectrofluormetry experiments carried out in this thesis show the mean $[Ca^{2+}]_i$ release of a population of neutrophils. In order to examine that neutrophil responses are similar across the cell population and there are synchronous free Ca^{2+} changes in individual neutrophils stimulated by PAF, single cell imaging experiments could be carried out using confocal to perform real-time fluorescent imaging. Ca^{2+} signals can be monitored in individual neutrophils using ratio imaging of Fura-2AM. This technique has already been used to show how individual neutrophils respond to fMLP and LTB_4 (Davies *et al.*, 1991). A suitable experiment would be to record Fura-2-AM fluorescence levels over a time period of 2 minutes, with an image being taken every 20 seconds in approximately 30 individual cells each time. Changes in intracellular fluorescence would be measured and used to calculate the levels of $[Ca^{2+}]_i$ in individual cells at various time points. Another method used to measure $[Ca^{2+}]_i$ levels is flow cytometry. However, it would be very difficult to obtain accurate results from flow cytometry as there is a time delay of several seconds before the sample is loaded into the machine and read by the detectors. Therefore, this method would be difficult to adapt for the experiments in this thesis as it is the signalling events of the first 60 sec that may be critical to activate SOCE. It would also be interesting to use real-time fluorescent imaging to investigate the effect of pre-incubating neutrophils with soluble E-selectin, to determine whether every neutrophil was contributing equally to the prolonged calcium release and identify whether a sub-population of neutrophils was responsible for causing a delayed Ca^{2+} signal in response to PAF which causes the prolonged Ca^{2+} release. However, all our results so far suggest that the whole population of neutrophils are acting synchronously. Importantly, in every whole cell patch clamp experiment the neutrophil responded to stimulation by PAF in a similar manner each time, which is consistent with this suggestion.

Receptor mediated activation of leukocytes by a number of inflammatory stimuli requires Ca^{2+} mobilization and influx as a critical common activation mechanism. Selective inhibition of distinct components of these Ca^{2+} signals may represent potentially attractive strategies for developing anti-inflammatory drugs to attenuate

leukocyte activation. Data presented in this chapter represents the first demonstration of soluble E-selectin causing permissive SOCE to occur following activation of neutrophils by PAF, and that this SOCE most likely occurs through TRPC6. This novel mechanism of molecular crosstalk relies on signals generated by a pertussis toxin-insensitive G_q protein being permitted to communicate with a TRPC which has been primed to receive its signals and cause SOCE. The expression of Ca^{2+} sensors STIM1 and CRACM1 suggests that these may also be involved in the SOCE pathway in human neutrophils and may have a role in soluble E-selectin prolonged PAF induced Ca^{2+} mobilization. This is an important observation as at present the role of these proteins in myeloid cells remains to be defined but it is clear that they are key players in SOCE mechanism and the focus of future research. The precise order of molecular events is yet to be fully explored (See figure 3.17). These observations raise the interesting possibility that modulation of TRP channel function may represent a novel therapeutic target for selective manipulation of pro-inflammatory neutrophil functional responses.

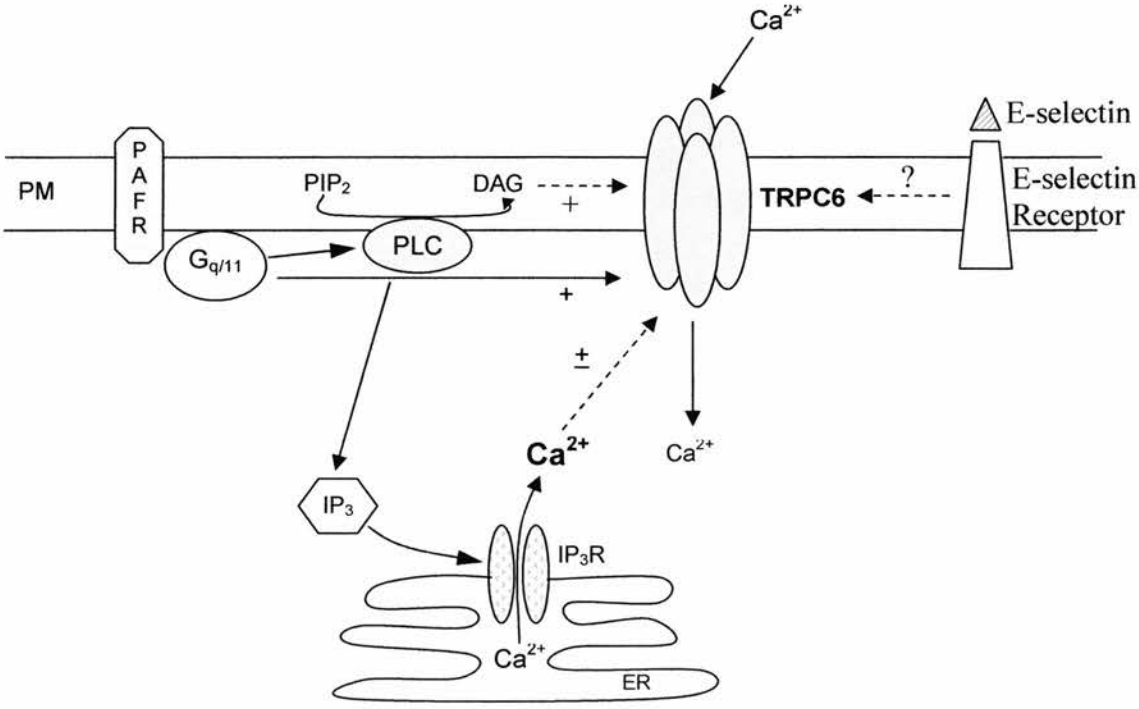


Figure 3.17 Schematic model of intracellular communication between E-selectin receptor and PAF receptor

PAF binds to its $G_{q/11}$-protein coupled receptor resulting in activation of PLC, leading to cleavage of PIP_2 and generation of membrane-retained DAG and cytosolic IP_3. DAG can directly activate TRPC6 (Li *et al.*, 2002). Soluble IP_3 activates the IP_3R on the endoplasmic reticulum to release intracellular Ca^{2+}. These responses result in the initial rapid increase of $[Ca^{2+}]_i$. E-selectin interacts with a receptor on the neutrophil surface, permitting PAF-induced Ca^{2+} mobilization to communicate with TRPC6 and allows "permissive" SOCE to occur. G = G-protein coupled receptor.

Chapter 4: Regulation of E-selectin mediated SOCE

4.1 Introduction

Recent investigations have extensively studied the regulation of TRPC channel activity. However, the exact mechanisms and components that activate and regulate TRPCs are still unknown. It is apparent that G-protein-coupled pathways linked to stimulation of PLC activity have a profound effect on regulating channel activity and that generation of DAG is a central event in these signalling cascades. DAG is a triglyceride that has a pivotal role in gating cation channels via both PKC-dependent and -independent mechanisms. Although production of DAG is pivotal to the activation of many of these channels, the pathways leading to generation of DAG and mechanisms by which DAG regulates opening of channels varies for different classes of ion channels.

PKC activation

The PKC pathway is a major signal transduction system that is activated upon stimulation of transmembrane receptors by hormones, neurotransmitters and growth factors. Key mediators of this pathway are formation of DAG and increased $[Ca^{2+}]i$ concentrations. The rise in $[Ca^{2+}]_i$ combined with the production of DAG causes activation and translocation of PKC to the plasma membrane where it can phosphorylate specific effector proteins and enzymes.

PKC comprises a family of serine/threonine kinases, which are activated principally by signals generated following receptor-mediated hydrolysis of membrane phospholipids (Nishizuka, 2003). PKC isoforms have been divided into 3 classes which are classified as calcium-dependent, calcium independent and atypical isozymes according to their sensitivity to Ca^{2+} and DAG. The conventional or Ca^{2+}-dependent PKCs (cPKCs: α, β and γ) require additional free Ca^{2+} and DAG for complete activation. The calcium-

independent novel PKCs (nPKCs: δ, ε, η and θ) are fully activated by DAG only, whereas the atypical PKCs (aPKCs: ζ, ι and λ) are Ca^{2+}- and DAG-independent (Newton, 2003). Activation of PKC results in the translocation of the enzyme from the cytosol to membrane compartment where it exerts its function (Nishizuka, 1995). Despite the extensive sequence and structural homologies between different PKC isozymes, especially within the catalytic domain, individual PKCs mediate unique cellular functions (Dempsey *et al.*, 2000; Jaken and Parker, 2000), presumably determined by the substrates that they phosphorylate (Carpenter *et al.*, 1987; Parker *et al.*, 1989)

Several proteins have been found to interact with PKCs with isoform selectivity (Poole *et al.*, 2004). For example among the PKC-interacting proteins, scaffolding/adaptor proteins termed RACKs (receptor for activated C kinases), bind with isozyme selectivity to PKC only after activation and translocation of the enzyme close to its relevant substrates (Mochly-Rosen *et al.*, 1995). Two RACK proteins have been identified and characterized namely RACK1 which is selective for PKCβ and RACK2 which is selective for PKCε (Schechtman and Mochly-Rosen, 2001). Localization of activated PKC isozymes to different subcellular sites confers specific substrate phosphorylation and different cellular responses. Therefore, binding to its respective RACK can localize each PKC isozyme next to a subset of protein substrates and away from others, thus conferring different functional specificity for each activated isoform (Mochly-Rosen *et al.*, 1995). Specific PKC isozymes can associate with a range of cytoskeletal proteins including myristoylated alanine-rich C-kinase substrate (MARCKS) and pleckstrin which are prominent substrates of PKC in many cell types (Hartwig *et al.*, 1992; Brumell *et al.*, 1997). Another protein suggested to be involved in the regulation of PKC is the 14-3-3 family of proteins (Aitken *et al.*, 1995; Aitken, 1996). Although 14-3-3 proteins are thought to play important roles generally in the regulation of PKC, it is not clear whether these two proteins interact *in vivo* and what the nature of this interaction is.

Phosphorylation of TRPC channels

Apart from the regulation of TRPC channels by DAG generated from PLC stimulation through either GPCRs or receptor tyrosine kinases (RTKs), little is known about the ability of additional signalling pathways downstream of receptor-stimulation to regulate TRPC activity. Activation via GPCRs or RTKs results in the rapid phosphorylation of many cellular proteins (Tsai *et al.*, 1997; Babnigg *et al.*, 2003). There are multiple potential phosphorylation sites for either serine/threonine kinases or tyrosine kinases in the primary sequence of all TRPCs. It is now clear that critical regulatory elements that control TRP channel activity include phosphorylation and Src family tyrosine kinases (Hisatsune *et al.*, 2004). However, few studies have specifically addressed the role of specific protein kinases in the regulation of TRPC channel activity.

Previous experiments have demonstrated that Ca^{2+} mobilization in neutrophils, an early key event in the control of motility, respiratory burst and degranulation, is prolonged in the presence of PAF and soluble E-selectin (Ruchaud-Sparagano *et al.*, 2000). PAF induces an increase in $[Ca^{2+}]_i$ which occurs primarily through Ca^{2+} release from IP_3-sensitive intracellular stores followed by an influx of extracellular Ca^{2+} via ion channels expressed in the plasma membrane. However, the mechanism for this prolonged Ca^{2+} response is not well defined. There have been a number of recent studies investigating the mechanisms by which selectins activate signalling pathways. For example, it has been reported that engagement of selectin receptors activates the MAPK pathway and/or activation of cell surface receptor-associated protein tyrosine kinases (Simon *et al.*, 2000). It has also been shown that Src family tyrosine kinases are involved in the induction of monocyte chemotaxis by soluble E-selectin (Kumar *et al.*, 2001), suggesting these would be pertinent mechanisms to investigate.

Membrane phospholipids are known to influence the activity of several types of ion channels, including TRP non-selective cation channels (Tong *et al.*, 2004). PI 3-kinase is a lipid kinase that phosphorylates phosphatidylinositol 4-phosphate and $PtdIns(4,5)P_2$

at the D-3 position producing PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Fruman *et al.*, 1998). Production of these phospholipids via PI 3-kinase signalling and subsequent activation of a downstream kinase cascade is critical for many cellular processes, including proliferation and protein synthesis. One of the best characterized targets of PI 3-kinase products is the protein kinase Akt (also known as protein kinase B) (Hanada *et al.*, 2004). In quiescent cells, Akt resides in the cytosol in a low-activity conformation. Upon cellular stimulation Akt interacts with PI 3-kinase generated phospholipids, causing its translocation to the plasma membrane, where it is phosphorylated and activated by 3'-phosphoinositide-dependent kinase 1 (PDK1) and 3'-phosphoinositide-dependent kinase 2 (PDK2) (Fresno Vara *et al.*, 2004). PI 3-kinase signalling regulates several types of ion channels, via modulation of vesicular movement and/or channel synthesis (Macrez *et al.*, 2001). For example, it was reported that growth factor stimulation requires PI 3-kinase for the rapid translocation of the TRPC5, from vesicles to the plasma membrane (Bezzerides *et al.*, 2004) and it has been shown that a TRPV1 and PI 3-kinase signal transduction complex facilitates trafficking of TRPV1 to the plasma membrane (Stein *et al.*, 2006). However, the phospholipid products of PI 3-kinase, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 are not themselves widely considered to be direct modulators of ion channel activity.

The results in Chapter 3 demonstrated that E-selectin mediated prolongation of PAF-induced Ca^{2+} mobilization was PLC-dependent. It is well established that PLC activation can lead to tyrosine phosphorylation of multiple proteins through downstream activation of tyrosine kinases. In this chapter, experiments were carried out to investigate the role of tyrosine kinases and PKC in regulating E-selectin induced permissive SOCE and to further elucidate the molecular mechanisms, which regulate TRPC6 channels in neutrophils.

4.2 Results

E-selectin-induced SOCE is regulated by Src and PI 3-Kinase

Specific protein kinase inhibitors were used to test the involvement of tyrosine kinases in soluble E-selectin-mediated SOCE. Inhibition of p38 MAP kinase by SB203580 (10μM) or the negative control SB202474 (10μM) had no effect on soluble E-selectin-induced Ca^{2+} influx following stimulation of neutrophils with PAF (Figure 4.1B). Similarly, a lack of effect of PD98059 (10μM) on soluble E-selectin-induced modulation of Ca^{2+} influx identified that MEK1 was not involved in mediating these responses (Figure 4.1B). However, consistent with published data (Hisatsune *et al.*, 2004), the specific Src family tyrosine kinase inhibitor PP2 (5μM) selectively inhibited the soluble E-selectin-induced SOCE to control levels of PAF-only stimulated neutrophils (Figure 4.1A and B). This effect was specific as the inactive analogue, PP3, had no effect on soluble E-selectin-induced Ca^{2+} influx. These data suggest either a direct role for Src in modulating TRPC6 channel activity or a role for Src in the downstream signalling events following soluble E-selectin binding to its putative receptor on neutrophils.

Western blot analysis of neutrophil protein lysates using a phosphorylation state-specific antibody (Tyr(P)116), which correlates with Src tyrosine kinase activation, showed significant phosphorylation above control levels with soluble E-selectin alone treatment (Figure 4.2A). Interestingly, stimulation of neutrophils with PAF alone had no effect on the levels of phospho-Src (Figure 4.2A). Pre-treatment of neutrophils with PP2, prior to stimulation with soluble E-selectin, inhibited active phospho-Src to below control levels (Figure 4.2A).

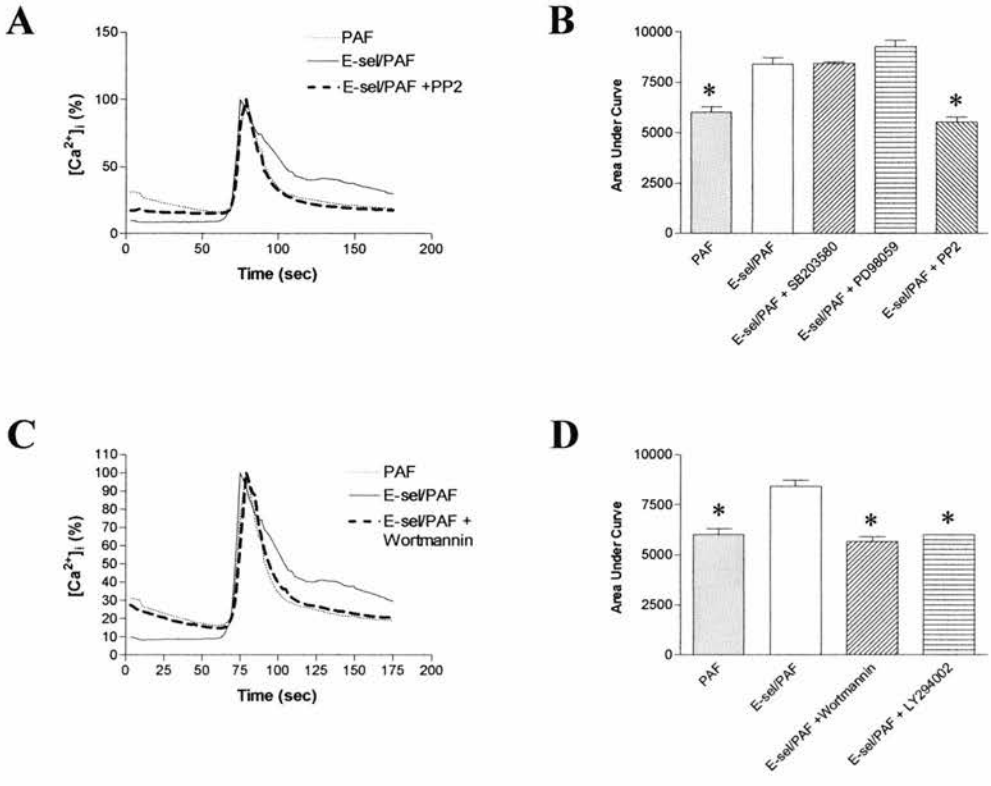


Figure 4.1 PI 3-Kinase and Src kinase activity are required for soluble E-selectin Ca^{2+} mobilization

(A) PD98059 (10μM), SB203580 (10μM) and PP2 (5μM) were added concurrently with E-selectin for 15mins prior to stimulation with 100nM PAF. The Ca^{2+} trace shown is representative of three separate experiments that were performed with similar results. (B) Bar graph representing area under the curves of graph A, calculated using GraphPad Prism software. Data expressed as mean ± sem of three independent experiments. (C) LY294002 (10μM, 5 min pre-incubation) and wortmannin (100nM, 15 min pre-incubation) were added prior to stimulation with 100nM PAF. The Ca^{2+} trace shown is representative of three independent experiments that were performed. (D) Bar graph representing area under the curves of graph A, calculated using GraphPad Prism software. Data expressed as mean ± sem from three separate experiments is shown. * Statistically different ($P<0.05$) from PAF/E-selectin-treated controls.

Since PI 3-kinase is also known to be a key regulator of ion channels in a variety of other cell types, neutrophils were pre-treated with the specific PI 3-kinase inhibitors wortmannin (100nM) or LY294002 (10μM) and LY303511 (10μM), an inactive structural analogue. LY303511 had no effect on soluble E-selectin-induced Ca^{2+} influx (data not shown). In contrast PI 3-kinase inhibition by either LY294002 or wortmannin inhibited the soluble E-selectin-induced SOCE to control levels (Figure 4.1C&D), thus identifying PI 3-kinase as an additional key regulator in the signalling pathway which mediates the effects of soluble E-selectin on Ca^{2+} influx.

Furthermore, LY294002 (10μM) had no significant effect on soluble E-selectin-induced phosphorylation and activation of Src (Figure 4.2A), indicating that PI 3-kinase may be involved in a parallel pathway or act downstream of Src in regulating Ca^{2+} influx. Treatment of neutrophils with soluble E-selectin caused activation of PI 3-kinase as assessed by a significant increase in phosphorylated Akt, compared with control untreated cells, whereas PAF did not induce any Akt phosphorylation (Figure 4.2B). Soluble E-selectin-induced increases in phospho-Akt levels could be inhibited completely by LY294002, confirming that it is a target of PI 3-kinase. Interestingly, the Src tyrosine kinase inhibitor PP2 also produced complete inhibition of phospho-Akt accumulation following treatment with soluble E-selectin (Figure 4.2B).

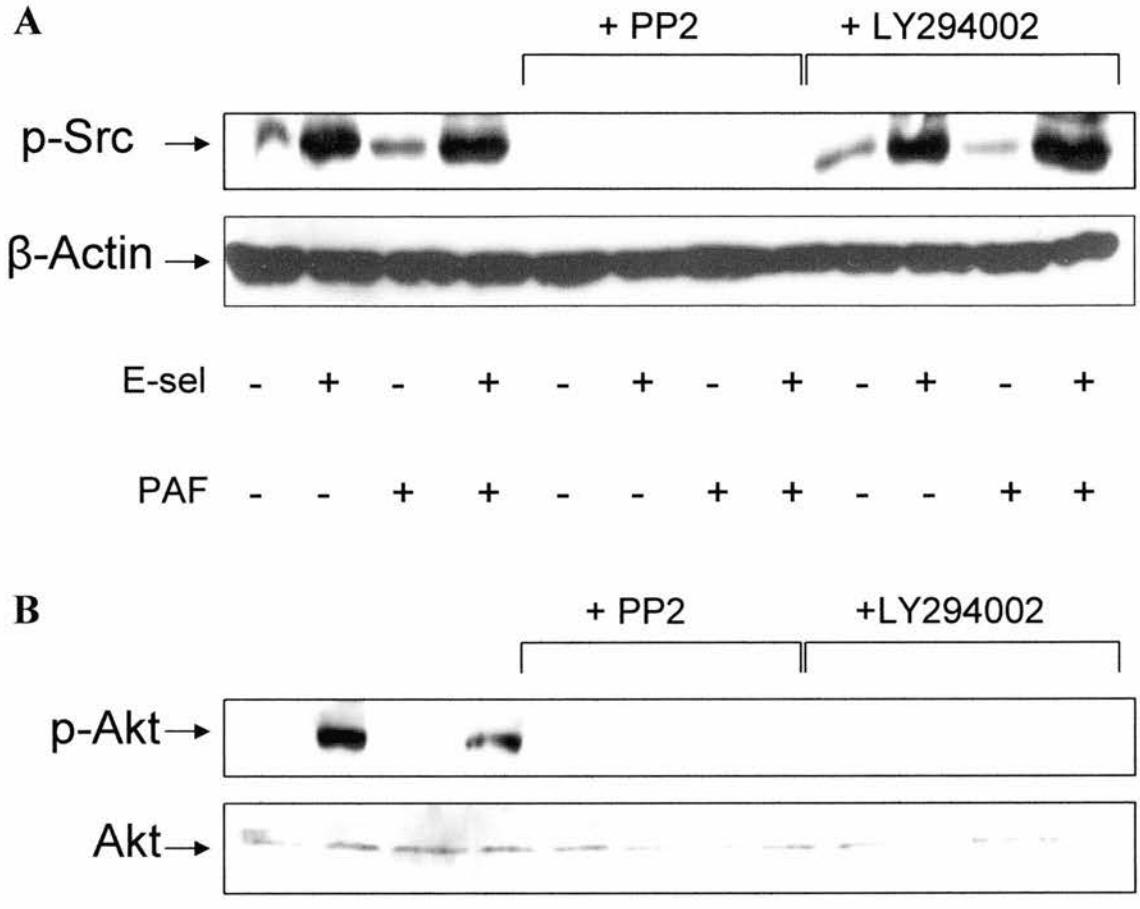


Figure 4.2 Soluble E-selectin induces Src & Akt activation in neutrophils

Freshly isolated neutrophils were incubated in the presence or absence of PP2 (5μM, 15 min) or LY294002 (10μM, 15 min), with soluble E-selectin (5μg/ml, 15min). Western blots of neutrophil lysates were carried out as described in Materials and methods, and probed with a (A) phospho-Src (Tyr 116) antibody (1:500) or (B) anti-phospho-Akt1/PKBα (Ser473) (1:200). To verify equal loading, the blots were probed with β-actin (1:10000) or total Akt (1:2000). This figure is a representative blot from 3 independent experiments that were performed with similar results.

PKC negatively regulates E-selectin mediated SOCE

Endogenous DAG undergoes continual turnover through the combined actions of DAG kinase and DAG lipase. Several groups have suggested that DAG is able to regulate TRPC ion channel activity (Hofmann *et al.*, 1999; Venkatachalam *et al.*, 2003). Therefore, to further investigate the role of DAG in TRPC activation, endogenous DAG levels were modulated by altering the function of DAG kinase, which actively converts DAG to phosphatidic acid. R59949 is an effective and specific inhibitor of Ca^{2+}-activated DAG-kinase (Jiang *et al.*, 2000), the catalytic function of which is very effective in reducing the elevated levels of DAG resulting from receptor-induced PLC activation (Jiang *et al.*, 2000). Firstly, effect of R59949 on Ca^{2+} mobilization induced by PAF or E-selectin and PAF was examined. As shown in figure 4.3A and B, PAF-induced Ca^{2+} mobilization was not affected, even at high concentrations (10μM) of R59949, whereas E-selectin prolonged Ca^{2+} mobilization following PAF treatment was sensitive to inhibition of DAG kinase (also see Figure 4.4). Inhibition of DAG kinase would be predicted to result in prolonged elevation of levels of DAG, activating PKC and possibly suggests that PKC activity may negatively regulate TRPC activity. These results could also suggest that elevated DAG levels may be involved in TRPC regulation. Experiments in Chapter 3 (Figure 3.12 and 3.13) showed that a DAG anologue (OAG) can cause Ca^{2+} influx but only when the TRPC channel is first 'primed' by E-selectin or store depletion.

To evaluate a role for PKC in regulating TRPC6 channels, we utilized Ro31-8220, which is recognized as a selective and potent inhibitor of multiple PKC subtypes (Keller and Niggli, 1993). Figure 4.5 demonstrates that the PKC inhibitor, Ro31-8220 (10μM) did not inhibit soluble E-selectin-induced modulation of Ca^{2+} influx following stimulation of neutrophils with PAF. Interestingly, Ro31-8220 significantly enhanced E-selectin-induced SOCE ($P<0.001$).

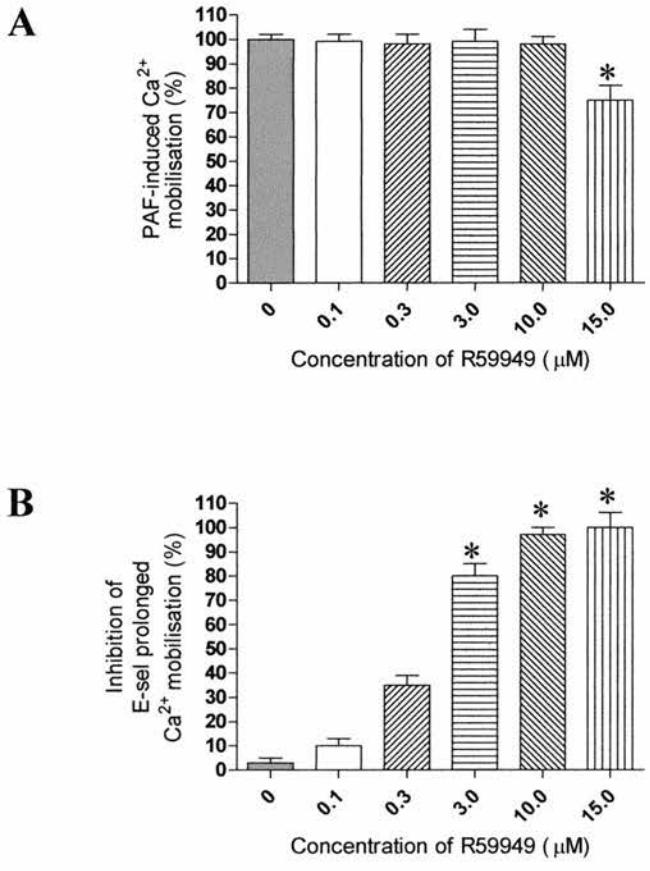


Figure 4.3 Effect of R59949, a DAG kinase inhibitor, on PAF-induced Ca^{2+} mobilization

(A) Concentration-dependent effect of R59949 on PAF-induced Ca^{2+} mobilization, freshly isolated neutrophils were loaded with Fura-2-AM (2μM) and preincubated with various concentrations of R59949 for 5 min before stimulation with 100nM PAF. (B) Concentration-dependent effect of R59949 on E-selectin prolonged PAF induced Ca^{2+} mobilization, freshly isolated neutrophils were loaded with Fura-2-AM (2μM) and preincubated for 15 min with soluble E-selectin (5μg/ml) and with various concentrations of R59949 for 5 min before stimulation with 100nM PAF. Data from three separate experiments that were performed are expressed as mean area under the curve ± sem. * Statistically different ($P<0.05$) from R59949 untreated controls.

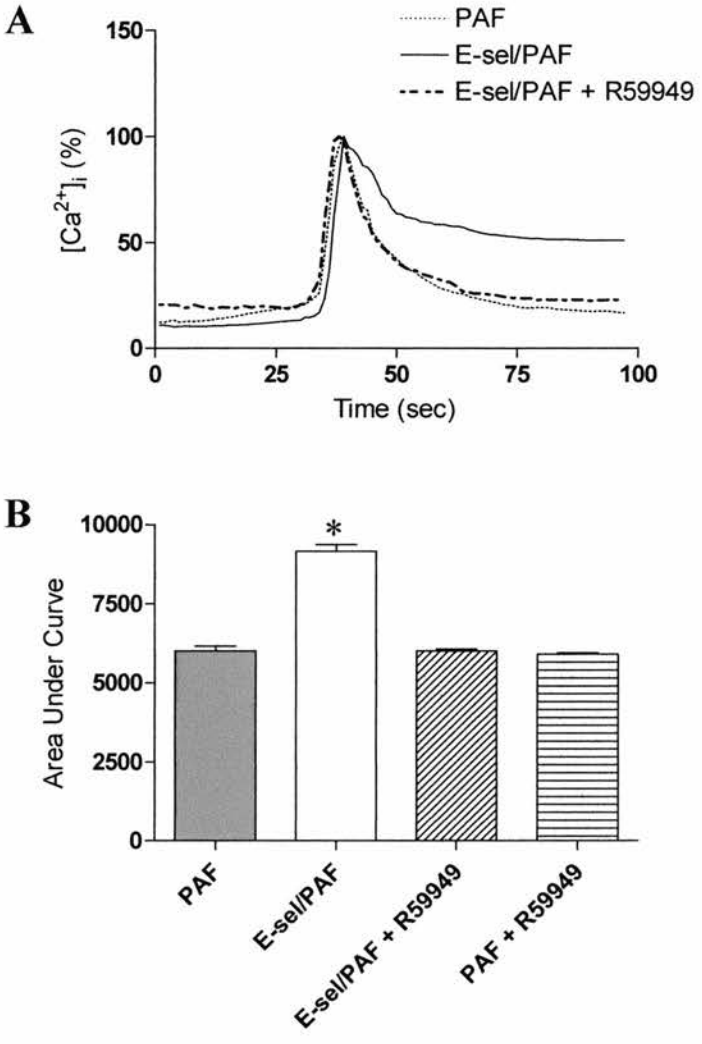


Figure 4.4 DAG kinase activity is required for soluble E-selectin Ca^{2+} mobilization
(A) Freshly isolated neutrophils were loaded with Fura-2-AM (2μM) and preincubated with or without E-selectin (5μg/ml) for 15 min and/or with 10μM R59949 for 5 min before stimulation with 100nM PAF. (B) Bar graph representing the area under the curves of the graph in A and a PAF + R59949 control from the same experiment (not shown on A), calculated using GraphPad Prism software. Data shown are expressed as mean area under the curve + sem from three independent experiments. * Indicates statistically different ($P<0.05$) from PAF treated controls.

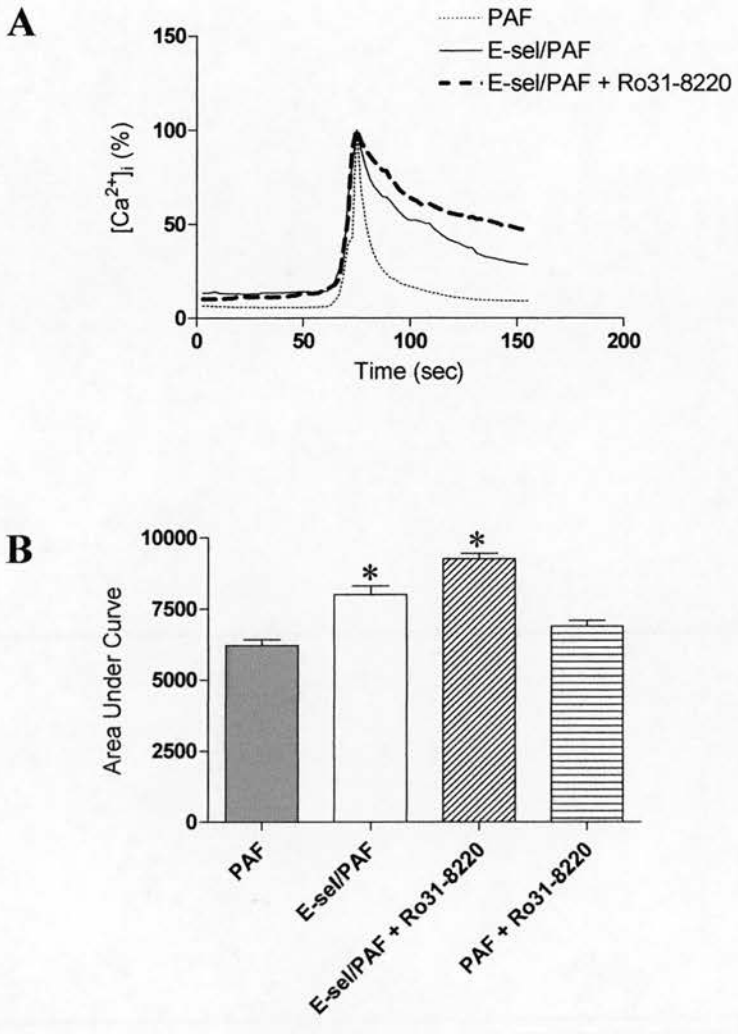


Figure 4.5 PKC is required for soluble E-selectin Ca^{2+} mobilization

(A) Freshly isolated neutrophils were preincubated with or without E-selectin (5μg/ml) for 15 min or with Ro31-8220 (1μM) for 5 min before stimulation with 100nM PAF. A representative Ca^{2+} trace from three separate experiments that were performed. (B) Bar graph representing area under the curves of the graph in A plus a PAF + Ro31-8220 control from the same experiment, calculated using GraphPad Prism software. Data shown are expressed as mean $\pm$ sem from three separate experiments. * Indicates statistically different ($P<0.05$) from PAF treated controls.

These data suggest either a direct role for PKC in negatively modulating TRPC6 channel activity or alternatively a role for PKC in the downstream signalling events following soluble E-selectin binding to its putative receptor on neutrophils.

In order to examine a means of activating PKC that was independent of DAG, the direct PKC-activator PMA was utilized, which causes pronounced PKC-mediated phosphorylation of targets at nanomolar levels (Quest *et al.*, 1997). PMA (100nM) selectively inhibited the soluble E-selectin-induced SOCE to control levels of PAF-only stimulated neutrophils (Figure 4.6) and the PAF-induced Ca^{2+} release from intracellular stores was unaffected. Taken together these data show that PMA-induced PKC activation inhibits channel activity and PKC inhibitors can potentiate the effect of E-selectin to prolong PAF-induced Ca^{2+} mobilization, suggesting that PKC negatively regulates TRPC channels.

TRPC6 channels are phosphorylated by PKC

Three protein kinase C phosphorylation sites that are common to all three TRPs in the TRPC3, TRPC6 and TRPC7 subfamily have been identified as Thr^{573}, Ser^{617}, and Ser^{712}. A recent study identified that TRPC3 channels are phosphorylated by PKC on Ser^{712} in the carboxyl-terminal domain (Trebak *et al.*, 2005). Therefore, investigation of the role of PKC-mediated phosphorylation in E-selectin mediated SOCE was carried out using an antibody that specifically recognizes any PKC substrates containing phosphoserine residues such as TRPC channels. Figure 4.7A demonstrates that in whole cell neutrophil lysates, PMA induced significant serine phosphorylation of a protein at around 100kDa. Co-immunoprecipitation techniques were then used to obtain evidence for direct phosphorylation of TRPC6 by PKC. The ability of the phosphoserine/PKC substrate antibody to bind TRPC6 channels was examined before and after activation of PKC with phorbol ester (PMA). In figure 4.7B the upper blot shows that the anti-phosphoserine/PKC substrate antibody binds to TRPC6 channels immunoprecipitated from cells following exposure to PMA.

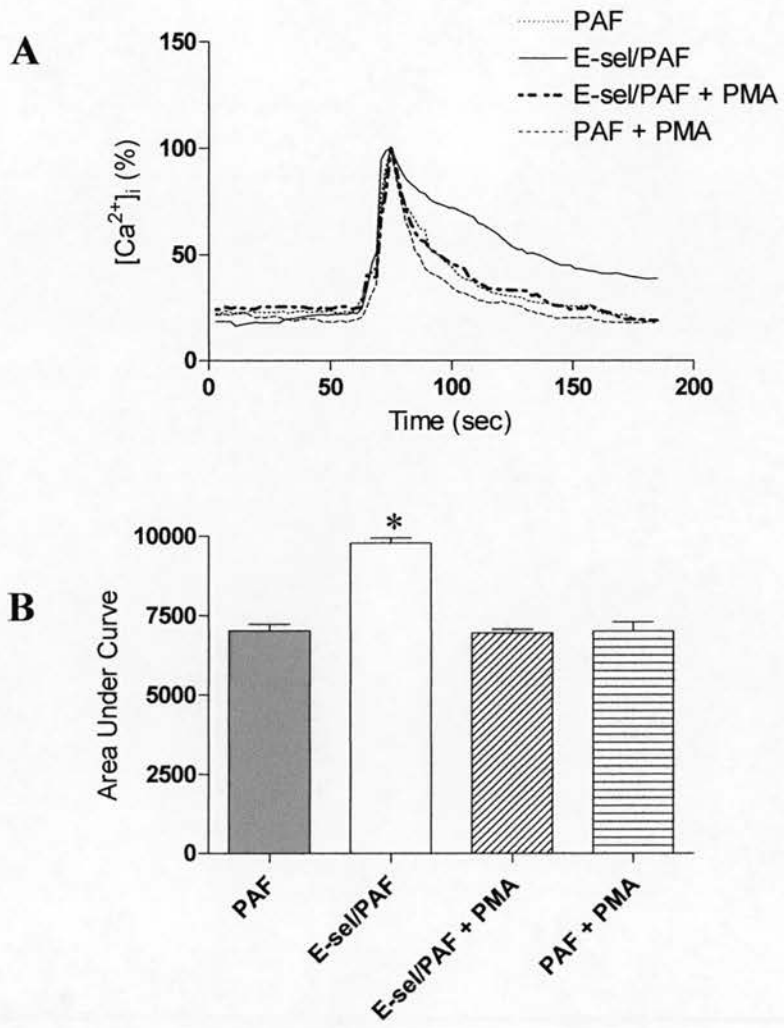


Figure 4.6 PKC activation with phorbol esters inhibits soluble E-selectin Ca^{2+} mobilization

(A) Ca^{2+} traces showing the effect of PMA on the prolonged $[Ca^{2+}]_i$ elevation induced by soluble E-selectin. 5µg/ml E-selectin was added 15 min and/or 100nM PMA was added 5 min before stimulation with 100nM PAF. The Ca^{2+} trace shown is representative of three separate experiments with similar results. (B) Bar graph representing area under the curves of graph C, calculated using GraphPad Prism software. Data shown is expressed as mean $\pm$ sem from three separate experiments that were preformed. * Indicates statistically different ($P<0.05$) from PAF treated controls.

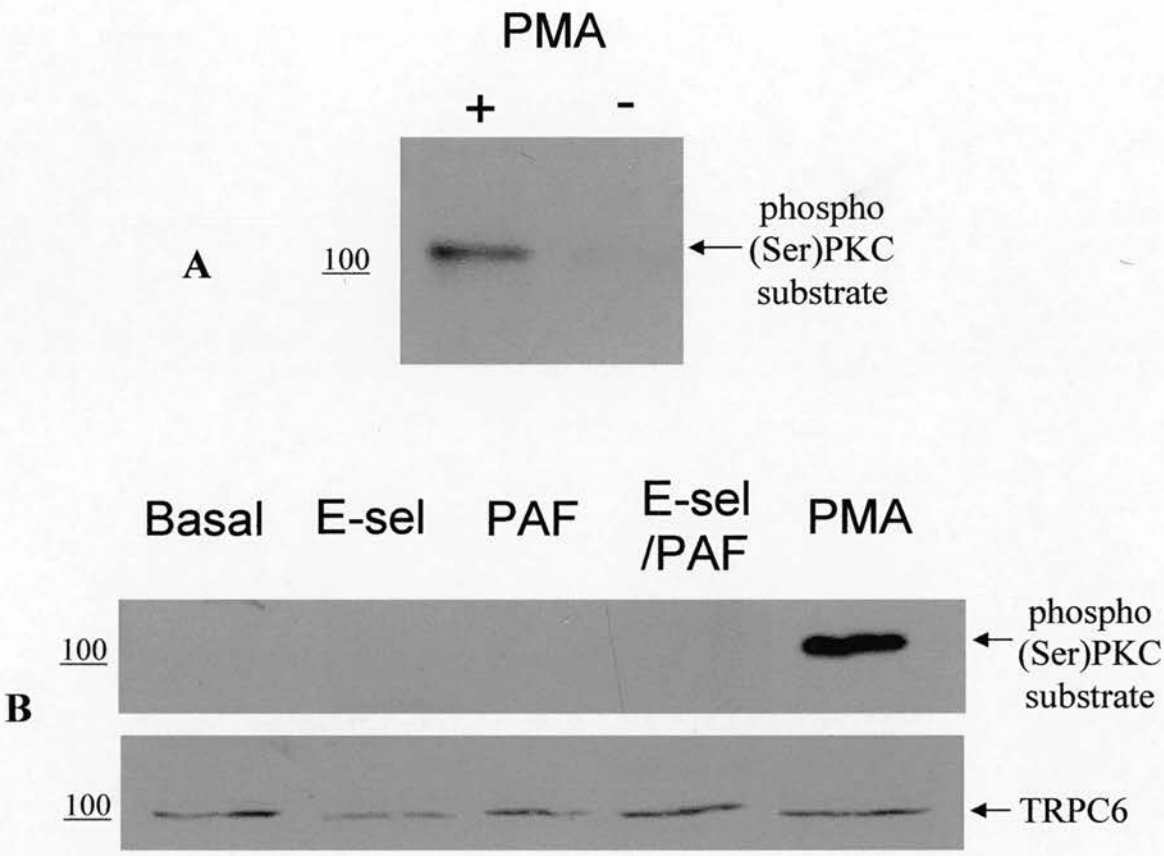


Figure 4.7 TRPC6 channels are phosphorylated following activation with PMA

(A) Western blotting of PMA (100nM for 5min) treated or untreated neutrophil membrane preparations probed with anti-phospho-Ser PKC substrate antibody (1:500) revealed a strong band at the predicted molecular weight (80kDa) as indicated. A representative immunoblot of three different experiments is shown. (B) Immunoprecipitation (IP) with anti-TRPC6 antibody and western blot analysis of the immunoprecipitates were performed as described in methods and materials. In the upper blot, phospho-Ser PKC substrates that coimmunoprecipitated with TRPC6 channels were detected by probing the transfer membranes with an anti-phospho-Ser PKC substrate antibody (upper blot). Immunoprecipitation of TRPC6 channels was confirmed by probing the same samples with an anti-TRPC6 antibody (lower blot). The results shown are representative of three independent experiments.

However, PAF and E-selectin did not induce phosphorylation of serine residues in TRPC6 (Figure 4.7B). The anti-phosphoserine/PKC substrate antibody was able to detect phosphorylated TRPC6 channels only from cells exposed to PMA and not from unstimulated cells or cells pretreated with E-selectin and/or PAF (Figure 4.7B).

PKC isoform activation

PKC predominantly resides in the cytosol in an inactive state and upon cell stimulation, translocates to the cytoskeleton or plasma membrane, where it becomes activated in the presence of specific lipid co-factors. As previous experiments have indicated a role for PKC in negatively regulating E-selectin prolongation of PAF-induced Ca^{2+} mobilization, experiments were carried out to investigate whether this effect was mediated by a specific subset of PKC isoforms.

Human neutrophils express the following PKC isoforms: PKCα, PKCβ, PKCδ, PKCι, and RACK1 a PKC-interacting protein, as detected by western blotting in whole cell lysates (Figure 4.8). The predicted molecular weights for each PKC isoform can be found in Table 4.1. Figure 4.8 shows several bands for the PKC isoforms present rather than one single distinct band, this is probably due to proteolysis of proteins by numerous highly active proteases present in neutrophils despite the presence of various protease inhibitors in the lysis buffer. Experiments investigating the location of these PKC isoforms and their translocation in response to different stimuli were carried out. In untreated neutrophils PKCα, PKCδ, PKCι and RACK1 were found in the Triton X-100 soluble fraction and no PKC isoforms were found in the Triton X-100 insoluble fraction (Figure 4.9). Colloidal gold staining confirmed that protein was present in the Triton X-100 insoluble fractions. Pre-treatment of neutrophils with soluble E-selectin had no effect on the presence or location of PKC isoforms (data not shown). However, in neutrophils pre-treated with soluble E-selectin and stimulated with PAF, both PKC isoform expression and localisation was altered.

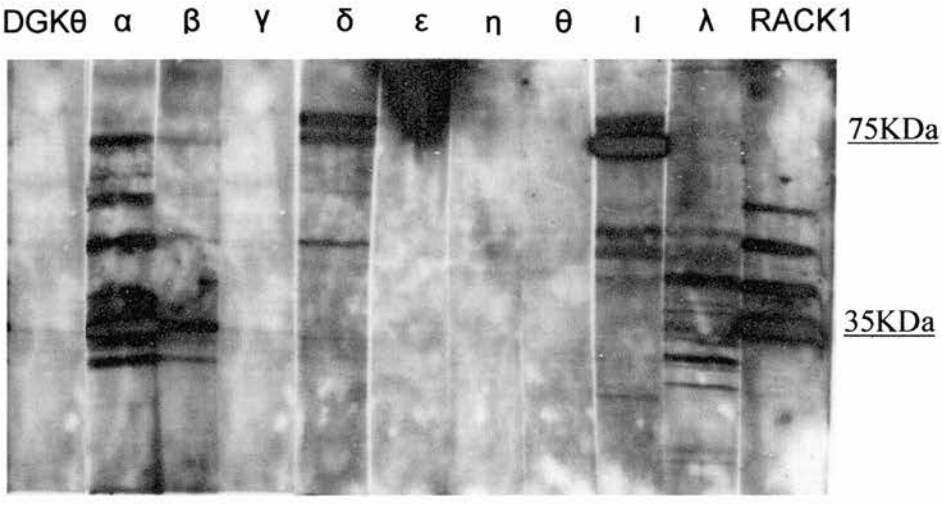


Figure 4.8 PKC isoforms present in whole cell lysates

Neutrophil lysates were carried out as described in Materials and Methods. Western blotting of neutrophil lysate fractions with PKC isoform antibodies (1:100) revealed bands for PKCα, β, δ, ι and RACK1 in human neutrophils. A representative immunoblot of three different experiments is shown.

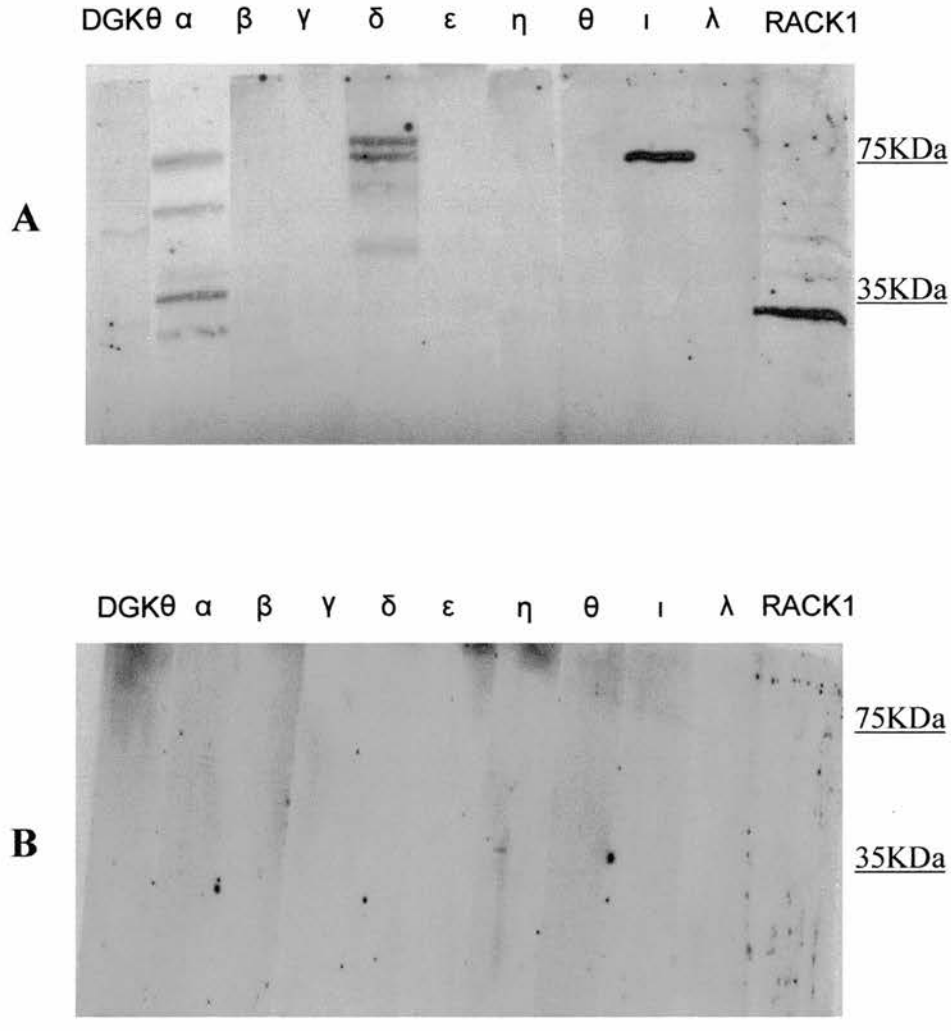


Figure 4.9 PKC isoforms present in untreated human neutrophils.

Triton X-100 soluble (A) and Triton X-100 insoluble (B) fractions of neutrophil lysates were carried out as described in Materials and Methods. Western blotting of neutrophil lysate fractions with PKC isoform antibodies (1:100) revealed bands for PKCα, δ, ι and RACK1 in the Triton X-100 soluble fraction of human neutrophils. A representative immunoblot of three different experiments is shown.

Under these conditions; PKCδ PKCι and RACK1 were found to be present in the Triton X-100 soluble fraction and PKCα, PKCβ, PKCδ and RACK1 were present in the Triton X-100 insoluble fraction (Figure 4.10). Treatment of neutrophils with PAF alone had the same PKC isoforms present in the Triton X-100 insoluble fraction but had different PKC isoforms present in the Triton X-100 soluble fraction, namely PKCβ, PKCδ, PKCι and RACK1. Table 4.1 summarises PKC isoform expression and location under the various treatments.

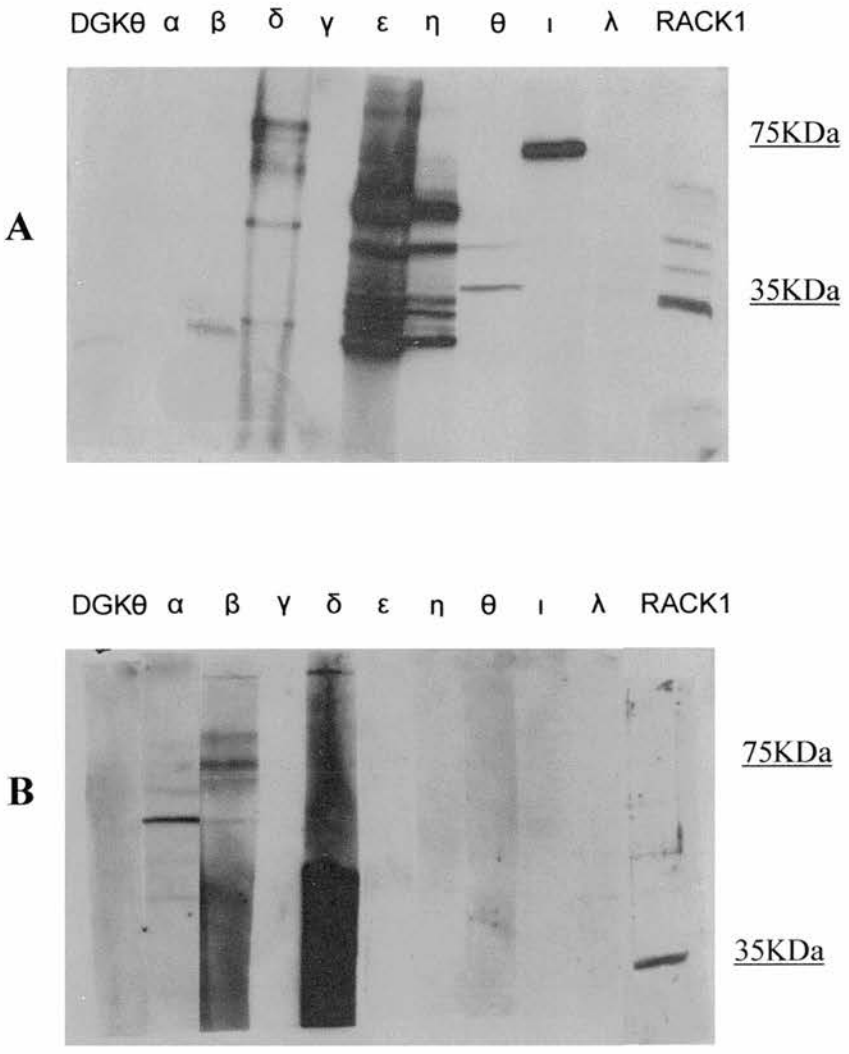


Figure 4.10 Soluble E-selectin and PAF induce PKC activation in Triton X-100 insoluble fraction of human neutrophils.

Freshly treated neutrophils were pre-incubated with soluble E-selectin (5μg/ml) for 15min before stimulation with 100nM PAF. Triton X-100 soluble (A) and Triton X-100 insoluble (B) fractions of neutrophil lysates were carried out as described in Materials and Methods. Western blotting of neutrophil lysate fractions with specific PKC isoform antibodies (1:100) revealed bands for PKCα, β, δ and RACK1 in the Triton X-100 insoluble fraction of human neutrophils. This figure is a representative blot from 3 independent experiments.

PKC Isoform		Basal		E-sel		PAF		E-sel/PAF	
Name	MW	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol
DKGθ	110								
PKCα	75	+		+			+		+
PKCβ	80					+	+		+
PKCγ	80								
PKCδ	78	+		+		+	+	+	+
PKCε	90								
PKCη	82								
PKCθ	79								
PKCι	74	+		+		+		+	
PKCλ	74								
RACK1	36	+		+		+	+	+	+

Table 4.1 Summary of PKC isotype expression in various neutrophil treatments

Sol = Triton X-100 soluble fraction, Insol = Triton X-100 insoluble fraction

4.3 Discussion

Despite the existence of multiple potential phosphorylation sites in the primary sequence of all TRPCs, few studies have specifically addressed the role of kinases in regulating TRPC channel activity. However, the possibility that tyrosine kinases might play a role in regulation of TRPCs has recently received some attention. Recent studies have shown that tyrosine phosphorylation by Src family protein tyrosine kinases represents a potential regulatory mechanism of TRPC6 channel activity (Trebak *et al.*, 2003; Hisatsune *et al.*, 2004). It has been suggested that two simultaneous events, coordinated opening of the channel by DAG and modulation by Src-induced tyrosine phosphorylation, contribute to the efficient influx of calcium through TRPC6 channels (Hisatsune *et al.*, 2004). We found that PP2, a selective Src inhibitor, caused specific inhibition of soluble E-selectin-mediated prolongation of PAF-induced SOCE compared to that observed in PAF-only stimulated neutrophils. In parallel, soluble E-selectin caused phosphorylation and activation of Src which was sensitive to inhibition by PP2 but was unaffected by PI 3-kinase inhibition with LY294002. These findings strongly suggest that Src activity is involved in modulating TRPC6 channel activity to regulate Ca^{2+} influx in human neutrophils.

Inhibition of PI 3-kinase also selectively blocked the soluble E-selectin-induced SOCE in neutrophils. Several potential intracellular regulatory motifs have been identified on TRPC6 including PI 3-kinase-SH2 recognition domains, suggesting a mechanism by which these channels might interact with the PI 3-kinase signalling pathway (Hofmann *et al.*, 1999). Several groups (Li *et al.*, 2002; Tseng *et al.*, 2004) have discovered that the PI 3-kinase lipid product PIP_3 can mediate Ca^{2+} influx through a mechanism independent of PLC activity and/or store depletion in several cell lines. Activation of receptor tyrosine kinase cascades leads to the membrane co-localization of PLCγ and PI 3-kinase, both of which utilise PIP_2 as a substrate to generate IP_3 and PIP_3 respectively. These two signalling intermediates trigger the activation of Ca^{2+} channels within different cellular compartments, giving rise to elevated levels of $[Ca^{2+}]_i$. Soluble E-

selectin was demonstrated to cause an increase in phospho-Akt, a downstream target of PI 3-kinase, suggesting that PI 3-kinase was activated following exposure of human neutrophils to E-selectin. Interestingly, phospho-Akt induced by E-selectin was blocked by PP2 which suggests that PI 3-kinase is regulated by Src and lies downstream of Src. Therefore, soluble E-selectin's ability to activate PI 3-kinase may be due to its ability to activate Src rather than to activate PI 3-kinase directly. Together, these data suggest a role for PI 3-kinase in modulating TRPC6 channel activity and that PI 3-kinase lies downstream of Src in the regulation of soluble E-selectin-mediated permissive SOCE.

To further define the regulatory role of the Src/PI 3-Kinase pathway in phosphorylation of TRPC6, immunoprecipitation and western blotting could be used together with the use of specific pharmacological inhibitors of these signalling pathways (PP2 to inhibit Src and LY294002 to inhibit PI 3-Kinase). Furthermore, the role of TRPC6 phosphorylation by Src family tyrosine kinases could be examined in neutrophils isolated from $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice (Meng and Lowell, 1997). As discussed in Chapter 3, using murine neutrophils limits the number of experiments using the methods for measuring $[Ca^{2+}]_i$ in this present study that can be carried out as typical yields from bone marrow neutrophil and peritoneal neutrophil preparations are approximately 1-2 million cells in total. However, alternative cell imaging techniques may make these measurements feasible in murine neutrophils.

Hoffmann *et al.* (1999) indicated that members of the subgroup TRPC3, TRPC6 and TRPC7 channels can each be activated in response to DAG through a mechanism independent of PKC. In the present study, experiments were carried out to investigate whether OAG, a DAG analogue, regulates TRPC activation. Results in Chapter 3 show that OAG does not directly activate TRPC channels in neutrophils (Figure 3.12). Further investigation of DAG-mediated regulation in neutrophils was carried out using R59949, an inhibitor of DAG metabolism, which inhibits DAG kinase and leads to an increase of DAG in the plasma membrane of the cell. Figure 4.4 shows that R59449 inhibited Ca^{2+} influx, suggesting that the relative levels of DAG have an important role in controlling

TRPC channel activity. It should be emphasized that the present experiments do not show that DAG directly opens the channel but that DAG is the single molecule identified so far that is closest to the channel.

Protein phosphorylation is not only important for activation mechanisms, but is also important for inactivation mechanisms of TRPC channels. It is well recognised that PKC can inhibit the activity of TRPC3 and TRPC6 (Zhang *et al.*, 2001; Trebak *et al.*, 2003) and that TRPC6 forms a multiprotein complex containing PKC as a regulatory component (Kim and Saffen, 2005). Experiments investigating the role of PKC in regulating TRPC6 channel activity indicated that activation of PKC, using phorbol esters (PMA), resulted in inhibition of E-selectin-mediated Ca^{2+} influx (Figure 4.6) whereas inhibition of PKC using Ro31-8220, a PKC inhibitor, enhanced Ca^{2+} influx (Figure 4.5). consistent with previous reports showing that TRPC3 can be phosphorylated by PKC resulting in a negative regulatory manner (Venkatachalam *et al.*, 2003). Overall, these results indicate that PKC may negatively regulate E-selectin induced SOCE through TRPC6.

An important area to investigate is the detailed molecular mechanism of PKC-mediated inhibition on TRPC channels. Recently, Trebak *et al.* (2005) showed that PKC inhibits TRPC3 proteins by directly phosphorylating Ser^{712} on the TRPC3 proteins. The mechanism by which this phosphorylation inhibits TRPC3 activity is not known but may cause a conformational change in the channel structure itself to limit ion movement or that phosphorylation of these key residues blocks a domain on the protein that interacts with a regulatory protein. These observations are consistent with a recent report showing that PKC phosphorylates TRPC6 channels on a conserved serine residue, Ser^{712}, in the carboxyl terminal domain (Kim and Saffen, 2005). Ser^{712} lies just downstream of the highly conserved TRP domain (Montell, 2001), a proline-rich sequence that is involved in the interaction of TRP proteins with regulatory proteins such as the immunophilin FK506 (Goel *et al.*, 2001; Sinkins *et al.*, 2004) and the scaffolding protein, Homer (Yuan *et al.*, 2003). By using antibodies that specifically

recognize phosphoserine residues in PKC substrates, experiments demonstrated that TRPC6 channels themselves are phosphorylated following exposure to PMA (Figure 4.7). In untreated and E-selectin treated cells, a low level of phosphoserine would be expected to be present to maintain the channel in an inactive state, which was not seen here. Other groups have also been unable to detect phosphoserine in untreated cells, which suggests that cellular levels may be below the limit of detection by immunoprecipitation in neutrophils. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis could be carried out to confirm that phosphorylation is present under unstimulated conditions. E-selectin and PAF treated samples may affect the phosphorylation of PKC sites on TRPC channel but again this is difficult to detect at a protein level in human neutrophils. However, the results show that protein kinase C activation by PMA induces TRPC6 phosphorylation and completely inhibits TRPC6 channel activity supporting the hypothesis that PKC negatively regulates TRPC6.

The data presented here also suggests that the negative regulation of TRPC6 channel represents a potential physiological feedback mechanism associated with G protein-coupled receptor stimulation and that protein kinase C plays an important role in this regulatory mechanism. Protein kinase C activators as well as compounds interfering with the sequence containing Ser^{712} might be useful in modulating TRPC6 activity and controlling Ca^{2+} entry into cells. Of course, identifying cell types in which TRPC6 is a component of native channels activated via specific physiological stimuli would be very useful in strategies aimed at selective control of Ca^{2+} homeostasis during disease. Understanding how the phosphorylation of Ser^{712} interferes with TRPC6 activation will help to unravel the molecular mechanisms of TRPC6 activation and shed light on how TRPC6 contributes to Ca^{2+} signalling in cells during physiological functions. To investigate the role of Ser^{712} in TRPC6 regulation it would be interesting to construct a site mutant in the PKC phosphorylation site of TRPC to confirm that PKC was negatively regulating TRPC6. In a recent study, mutation of Ser^{712} of TRPC3 to alanine

was shown to prevent PKC phosphorylation upon PMA stimulation (Trebak *et al.*, 2005), confirming that PKC directly phosphorylates TRPC channels.

Recently published studies have suggested that trafficking of TRPC channels is an important aspect of their regulation (Cayouette *et al.*, 2004; Bezzerides *et al.*, 2004; Morenilla-Palao *et al.*, 2004; Singh *et al.*, 2004). Therefore, it is possible that phosphorylation of these ion channels could prevent proper targeting to the plasma membrane or could cause channels to be internalized. To date, the specific PKC isoforms involved in regulating the phosphorylation and translocation of TRPC6 in intact human monocytes and neutrophils have not been identified. It was therefore important to investigate if E-selectin and PAF acted to recruit PKC isoforms to 'prime' the TRPC channel in neutrophils. Upon activation, PKC isoforms translocate from soluble to particulate compartments (plasma membrane, nucleus, and cytoskeleton), in a similar way as reported for TRPC channels (Cayouette *et al.*, 2004). A variety of PKC isoforms are found in human neutrophils (Kent *et al.*, 1996; Sergeant and McPhail, 1997). PKC is kept in an inactive "folded" conformation by binding of the pseudosubstrate sequence to the substrate binding site in resting cells (Tan and Parker, 2003). PKC becomes activated when conformational changes associated with phospholipid binding displace the pseudosubstrate moiety from the catalytic domain, thus allowing PKC to phosphorylate its protein substrates.

In this study, we examined the PKC isoforms present in Triton X-100 soluble and Triton X-100 insoluble fractions of human neutrophils by western blotting analysis with monoclonal antibodies against PKC isoforms and demonstrated that human neutrophils express at least two classical PKCs; α, β, one novel PKC, δ, and one atypical PKC; ι. (Figure 4.8). PKC α, β δ and ζ have been shown by numerous groups to be present in human neutrophils (Dang *et al.*, 1995; Kent *et al.*, 1996; Naucler *et al.*, 2002). The specificity and biological activity of PKC isoforms are regulated by their subcellular localization. Thus, after activation, PKC isoforms are often translocated to other compartments. Translocation of PKC is isoform-, cell type-, and activator-specific, and

is tightly regulated by various cofactors (Oancea *et al.*, 1998; Shirai *et al.*, 1998). Each PKC may, therefore, display a distinct subcellular localization and bind to intracellular proteins that serve as substrates and/or carriers, such as receptors for activated C kinases, receptors for inactive C kinases, MARCKs, annexins, and cytoskeletal components (Mochly-Rosen *et al.*, 1995). In resting neutrophils PKC α, β and δ are known to reside in the cytosol and upon activation they have been shown to translocate to membrane fractions (Bussolino *et al.*, 1994; Faghiri and Bazan, 2006). Soluble E-selectin alone had no effect on the distribution of the Triton X-100 soluble and insoluble PKC isoforms. However, cells stimulated with PAF or E-selectin/PAF induced a sustained and marked translocation of PKC α, β and δ from the Triton X-100 soluble to the Triton X-100 insoluble fraction.

The data shown here indicates that human neutrophils contain members of all three PKC isoform families and that translocation of cPKC and nPKC to the plasma membrane may occur in cells in response to PAF as well as to E-selectin/PAF. These results demonstrate that conventional PKCα and PKCδ translocate to the Triton X-100 insoluble fractions in response to PAF and E-selectin. However, these PKC isoforms also showed translocation following PAF treatment alone, suggesting that they may not be involved solely in regulating TRPC. No atypical PKCs moved to the Triton X-100 insoluble fraction upon stimulation which may be due to their Ca^{2+}- and DAG-independent activation mechanism (Newton, 2003). Unforutantely, in the experiments carried out in this thesis the Triton X-100 soluble and insoluble fractions probably contained a mixture of proteins, rather than representing distinct cytoplasmic and cytoskeletal fraction as orginially intended. The Triton X-100 soluble fraction probably contained membrane, cytoplasmic and some cytoskeletal proteins and the Triton X-100 insoluble may have contained nuclei and some cytoskeletal proteins. The speed of the first spin (14,000 g for 10min) in the Triton X-100 fractions protocol (see section 2.14) was too low to spin out all the cytoskeletal proteins present in neutrophils lysates. A spin of approximately 200,000 g for 40mins would be required to pull out all cytoskeletal proteins. Therefore, blotting for known cytoskeletal, cytosolic and membrane proteins

needs to be carried out to prove what is present in each fraction before definite interpretations of these results can be made. The protocol will need to be adapted to achieve distinct cytoplasmic and cytoskeletal fractions or ultracentrifugation of neutrophils could be carried out to fractionate out the cytoskeletal and cytoplasmic proteins if this work was continued.

Further studies are needed to determine if E-selectin and PAF recruits PKC isoforms to 'prime' TRPC channels for activation and if PKC membrane localization is required for TRPC6 activation. For example, PKC-mediated internalization or redistribution of fluorescently labelled TRPC6 channels could be examined using confocal microscopy when the appropriate antibodies become available.

The data presented in this chapter demonstrates that both Src and PI 3-kinase are required in a common pathway to mediate the soluble E-selectin-induced permissive SOCE and resulting prolongation of $[Ca^{2+}]_i$. These results also indicate that increased opening of TRPC6 by E-selectin is negatively regulated by PKC. Several other groups have also shown PKC to have a negative effect (Trebak *et al.*, 2003; Estacion *et al.*, 2004). Figure 4.12 shows a schematic model of the role of Src, PI 3-kinase and PKC phosphorylation in regulating E-selectin induced permissive SOCE by PAF. These results demonstrate that the Src, PI 3-kinase and PKC pathways mediate the soluble E-selectin-induced prolongation of $[Ca^{2+}]_i$ elevation, and raise the interesting possibility that modulation of TRP channel function may represent a novel therapeutic target for selective manipulation of potentially pro-inflammatory neutrophil functional responses.

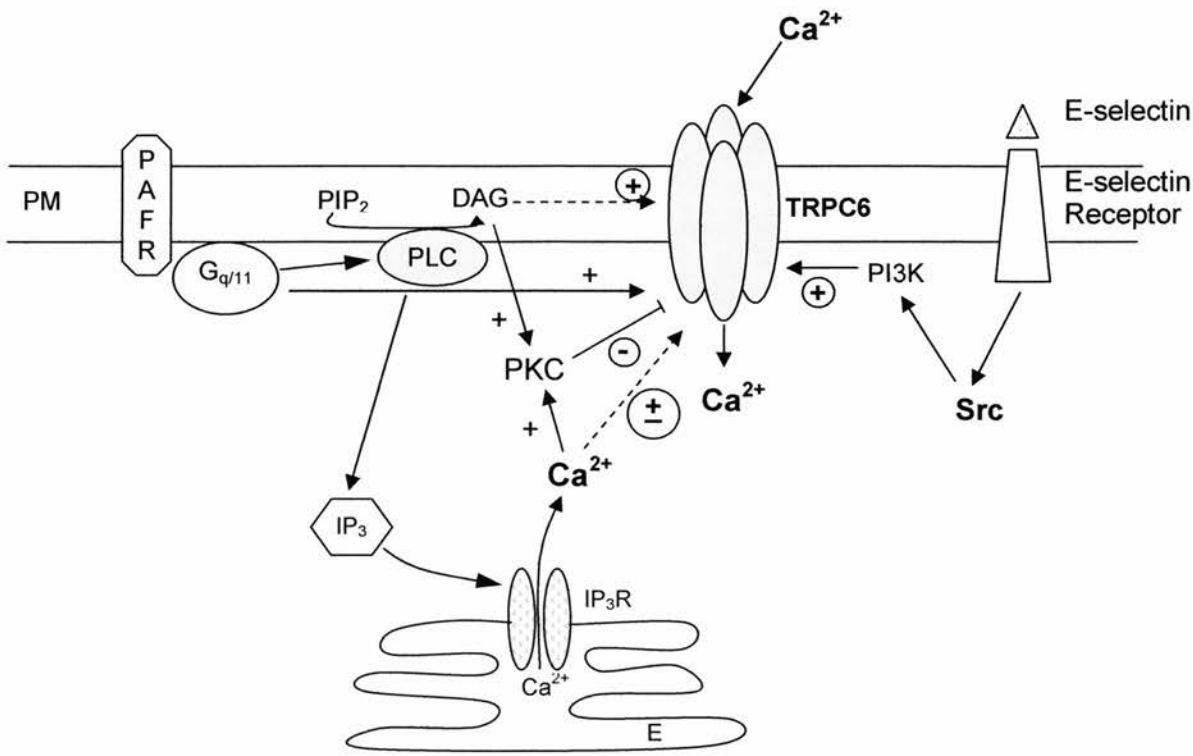


Figure 4.12 Schematic model of TRPC regulation

PAF binds to its $G_{q/11}$-protein coupled receptor resulting in activation of PLC, leading to cleavage of PIP_2 and generation of membrane-retained DAG and cytosolic IP_3. DAG can directly activate TRPC6 (Li *et al.*, 2002) and this study showed that DAG may possibly directly activate TRPC6 channel when it is primed. Positive modulation of TRPC6 channel involves Src and PI 3-kinase pathways and PKC negatively regulates TRPC6.

Chapter 5 – Functional Consequences of E-selectin mediated SOCE

5.1 Introduction

In neutrophils, Ca^{2+} signalling is important for a number of cellular activities, including adhesion, chemotaxis, phagocytosis, respiratory burst and apoptosis (Jaconi *et al.*, 1991; Dewitt and Hallett, 2002; Theander *et al.*, 2002; Niggli, 2003). The Ca^{2+} influx through SOCs into the cytosol is responsible for many of these neutrophil responses (Dillon *et al.*, 1988; Lee *et al.*, 2005). Therefore, it is important to understand the functional consequences of SOCE in neutrophils.

Adhesion

It has been suggested that the binding of neutrophil adhesion receptors plays an important role in the regulation of neutrophil function and that interactions which occur between neutrophils and endothelial cells via E-selectin during rolling adhesion may play a part in regulating subsequent adhesion events (Zimmerman *et al.*, 1992). The potential for fine regulation of neutrophil adhesion through the β_2 integrins is clear from studies that demonstrate functional modulation of these receptors (Hynes, 1992; Takagi and Springer, 2002). However, the interactions and physiological mechanism underlying control of neutrophil adhesion has not been fully defined. Several groups have reported that the selectin family may act to trigger β_2 integrin-mediated adhesion (Simon *et al.*, 1995; Bunting *et al.*, 2002). Ruchaud-Sparagano *et al.* (2000) showed that exposure of neutrophils to soluble E-selectin and PAF in combination induced a synergistic effect upon β_2-integrin-mediated adhesion.

Chemotaxis

One of the most important functions of neutrophils is to respond by chemotaxis to a variety of products released either by the host or by the invading organism (Babior *et al.*, 2002). Transmigration requires initial contact and adhesion of neutrophils to endothelial cells through cell adhesion molecules such as E- and P-selectin, followed by active, chemokine-directed migration of neutrophils through the endothelial cell layer to the site of injury or infection. Recent studies have suggested that soluble E-selectin is a potent chemotatic factor for monocytes and that soluble E-selectin mediates monocyte chemotaxis through Src family tyrosine kinases (Kumar *et al.*, 2001). However, the role of E-selectin in regulating chemotaxis in neutrophils remains to be fully investigated.

Superoxide Release and Priming

Superoxide production by neutrophils participates in the destruction of invading organisms and in host defence (Babior, 2000). The inappropriate production of superoxide can contribute to destruction of normal tissues and to the prolongation of the inflammatory process and therefore must be tightly controlled. For example as discussed in Chapter 1 (Section 1.3.1), CGD is a primary immunodeficiency that affects the oxidative mechanism of microbial killing of phagocytic cells and is characterized by a lack of, or severely reduced superoxide anion (O_2-) production by neutrophils (Barese *et al.*, 2005). CGD normally results from a gene mutation and/or abnormality of the NADPH oxidase components, particularly cytochrome b558 (gp91phox and p22phox) in the membrane and the cytosol factors p47phox, p67phox, p40phox and others present in neutrophils (Curnutte, 1992).

Neutrophil functional responses are subject to priming (Aida and Pabst, 1991) which is an alteration in the activation state of the cell that amplifies subsequent responses. Low-level *in vitro* exposure of neutrophils to priming agents such as PAF, LPS and TNFα, does not result in superoxide release but causes shape change, an increase in

adhesiveness and a decrease in deformability of the cytoskeleton and consequently chemotactic response. Primed neutrophils show an increased respiratory burst in response to stimulation with chemotatic factors such as fMLP. Neutrophil priming also leads to increased vascular sequestration and combinations of priming agents could act to amplify neutrophil-mediated damage to the endothelium both *in vivo* and *in vitro*.

Apoptosis

During *in vitro* culture, neutrophils undergo spontaneous apoptosis, with characteristic morphologic alterations (cytoplasmic and nuclear condensation) and intranucleosomal DNA cleavage (Savill *et al.*, 1989). Neutrophil apoptosis is associated with the marked down-regulation of a number of neutrophil functions, including phagocytosis, stimulated granule secretion and chemotaxis (Dransfield *et al.*, 1995), many of which show marked adhesion-dependency. One study characterizing apoptosis-associated cell surface changes showed that apoptotic neutrophils have a markedly reduced capacity to bind to E-selectin (Dransfield *et al.*, 1995). They suggest that this is a consequence of the reduced availability of the sialyl Lewis^x selectin ligands, which are down-regulated on the apoptotic cell surface.

E-selectin ligands

The first molecular contacts formed between the endothelium and circulating neutrophils occur via vascular E- and P-selectins expressed by activated endothelium, resulting in rolling adhesion which probably represents a key event in regulation of neutrophil function (Lawrence and Springer, 1993; Frenette *et al.*, 1996). Recent studies have focused on the identification and characterization of glyco-conjugate ligands for the selectins (Sperandio *et al.*, 2006). Several ligands for E-selectin, which all contain sLe^x-type glycans, have been identified including P-Selectin Glycoprotein Ligand-1 (PSGL-1) (Asa *et al.*, 1995), L-selectin (Picker *et al.*, 1991), CD66 (Kuijpers *et al.*, 1992), CD44 (Dimitroff *et al.*, 2001) and E-selectin ligand 1 (ESL-1) (Levinovitz *et al.*, 1993)).

It is probable that other Le^x and sLe^x expressing glycoproteins and glycolipids on the neutrophil are also capable of mediating interactions with the selectins. However, E-selectin ligands on neutrophils are less well characterized. PSGL-1, which is found on leukocytes and platelets, binds to P-, E- and L-selectins *in vitro* and represents an important functional ligand for all of these molecules (McEver and Cummings, 1997). Experiments carried out in Chapter 3 demonstrated that E-selectin binds via the lectin domain to a ligand present on neutrophils and was required for the prolongation of Ca^{2+} mobilisation observed in neutrophils in response to PAF. Furthermore, the results in Chapter 3 demonstrated that soluble E-selectin caused increased $[Ca^{2+}]_i$ release following stimulation with PAF. In this chapter, experiments were carried out to examine the functional consequence of this increased $[Ca^{2+}]_i$ release and the potential role of TRPC channels in mediating several important neutrophil functional responses. In addition, the role of the lectin domain in neutrophil responses was investigated and potential neutrophil E-selectin counter-receptor(s) were investigated.

5.2 Results

Recombinant E-selectin supports neutrophil adhesion

Figure 5.1 shows that recombinant E-selectin promoted neutrophil adhesion to E-selectin-coated wells, which was susceptible to inhibition by the monoclonal antibody ENA2 $F(ab')_2$, confirming the requirement of the lectin domain for E-selectin mediated adhesion.

Soluble E-selectin inhibits neutrophil chemotaxis towards PAF

Augmentation of β_2 integrin-dependent adhesion by soluble E-selectin (Ruchaud-Sparagano *et al.*, 2000) raised the possibility that soluble E-selectin may also affect the potential for β_2 integrin-mediated directed migration of neutrophils towards the chemoattractant PAF. A β_2 integrin blocking mAb (TS1.18) abolished migration towards PAF (Figure 5.2) confirming the β_2 integrin-dependent nature of neutrophil migration. Figure 5.2B shows that the PAF-directed migration of neutrophils was inhibited by soluble E-selectin, an effect which was reversed when soluble E-selectin was pre-incubated with ENA2. These data suggest that increased β_2 integrin-mediated adhesive interactions in the presence of soluble E-selectin acts to slow migration processes, prolonging neutrophil-endothelial cell interactions and may inhibit transmigration *in vivo*.

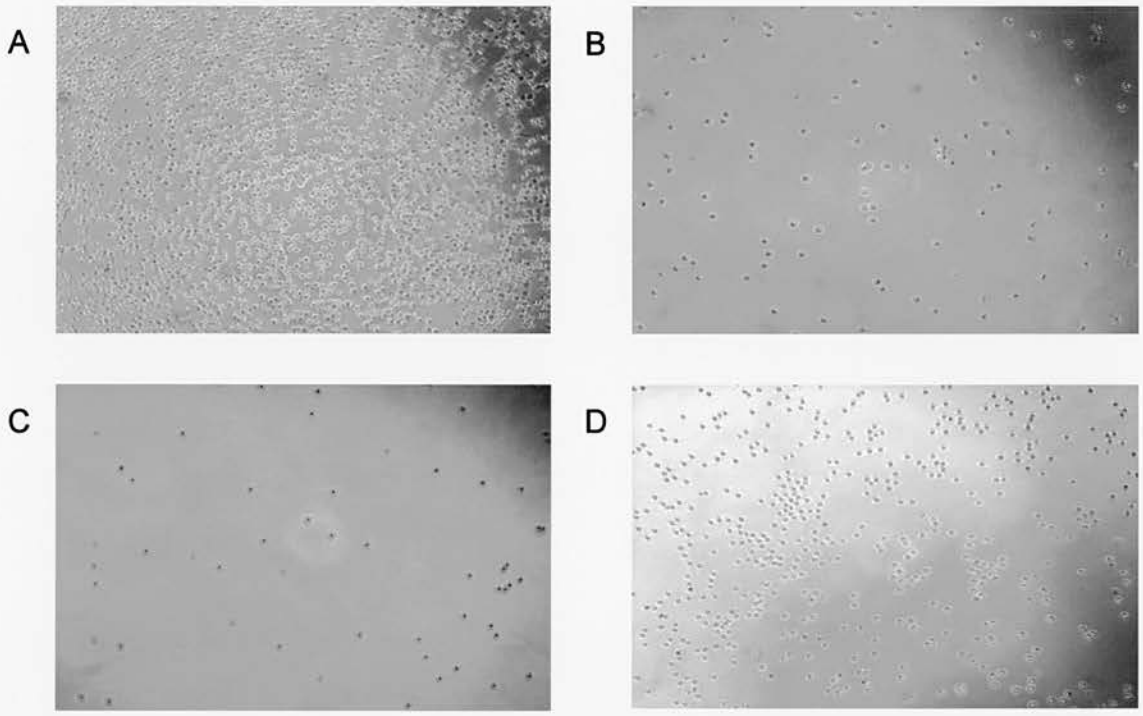


Figure 5.1 - Neutrophil adhesion to selectin coated plates shown by light microscopy

Plates were coated with soluble E-, P-selectin or FCS at a concentration of 1ug/ml overnight at 4°C. For adhesion blockade using CD62E mAb, wells were preincubated with 1:50 dilution of ENA2 $F(ab')_2$ for 30 min at 4°C. Wells were washed six times in assay medium alone and fixed with 3% paraformaldehyde and adhesion of neutrophils was viewed under a light microscope. A = E-selectin, B = CD62E blockade of E-selectin adhesion, C = FCS (negative control) & D = commercial recombinant E-selectin (positive control). All images taken with a Zeiss Axiovert s100 with 10x objective. Pictures are representative of three separate experiments.

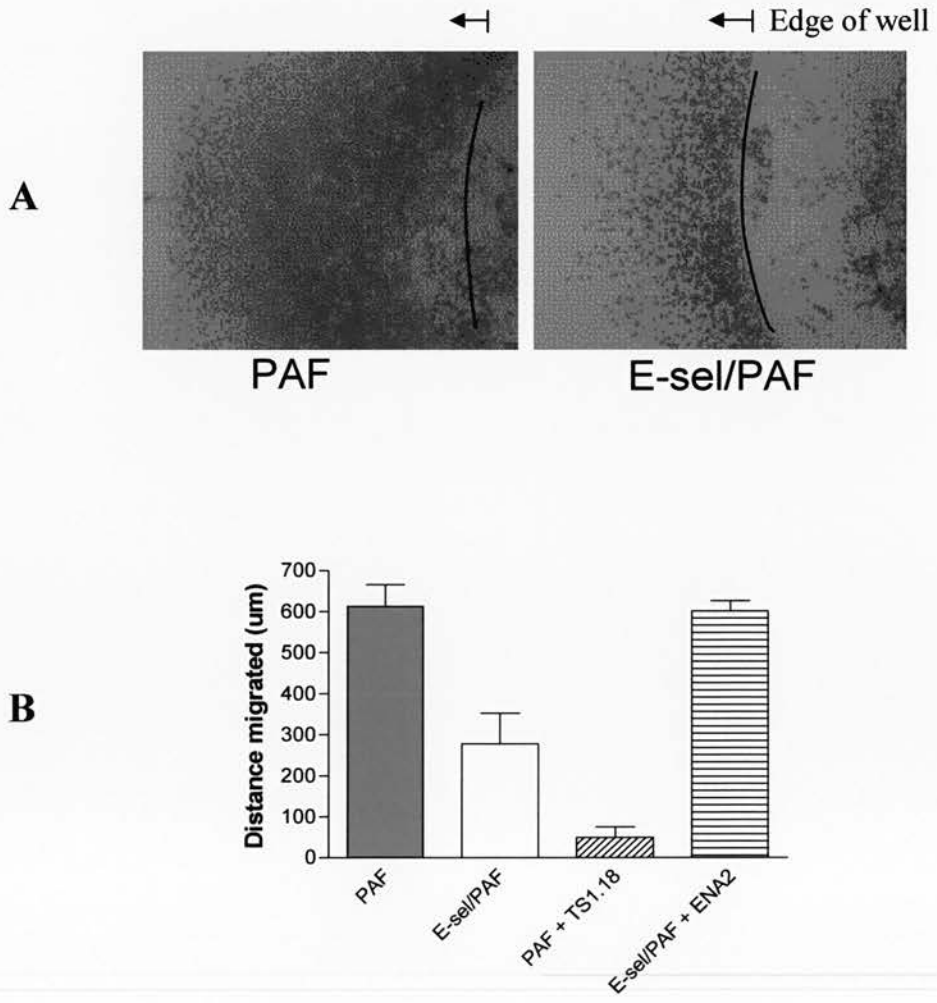


Figure 5.2 Soluble E-selectin inhibits neutrophil chemotaxis

Chemotaxis of freshly isolated neutrophils was assessed using sub-agarose method (see section 2.9). Figure A shows images taken with a Zeiss Axiovert s100 with 10x objective. After staining with Diff-Quick, the distance from the origin to the leading edge of migrating cells was determined using computer image analysis. Data shown are the mean $\pm$ sem from six separate experiments. E-selectin was used at 5µg/ml and TS1.18 an anti-CD18 monoclonal Ab was used at 10µg/ml. For blockade of CD62E, ENA2 $F(ab')_2$ (1:50) and soluble E-selectin were preincubated for 1 h.

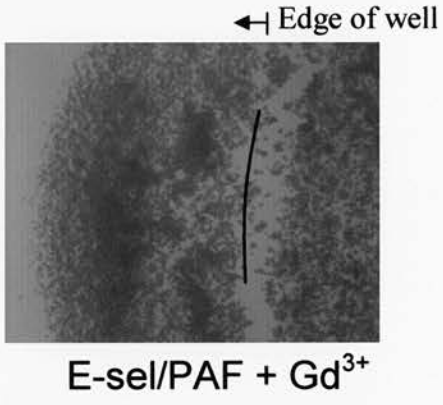
SOCE inhibition reverses E-selectin chemotaxis inhibition

Experiments were carried out to investigate the effect of Ca^{2+} channel inhibitors on E-selectin inhibition of PAF-directed chemotaxis. Figure 5.3, shows Gd^{3+}, a SOC and TRPC channel blocker, reverses the inhibitory effect of E-selectin on chemotaxis, suggesting a role for E-selectin-mediated SOCE in the inhibition of chemotaxis. As Gd^{3+} reverses the effects of E-selectin on chemotaxis, this would further suggest a role for TRPC channels being involved in this response.

Tyrosine phosphorylation reverses E-selectin effects on chemotaxis

The results in Chapter 4 (Figure 4.1) suggested a role for Src family tyrosine kinases in soluble E-selectin-induced SOCE. Kumar *et al.* (2001) have recently shown that Src family tyrosine kinases are involved in the induction of monocyte chemotaxis by soluble E-selectin. Therefore, experiments were carried out to investigate the involvement of tyrosine kinases in neutrophil chemotaxis. To further define the pathways involved in neutrophil activation following soluble E-selectin treatment, the effects of specific inhibitors of protein kinases involved in intracellular signaling were tested upon soluble E-selectin-induced inhibition of chemotaxis. Inhibition of E-selectin-mediated neutrophil migration in response to PAF was reversed by PP2, a Src family tyrosine kinase inhibitor (Figure 5.4), suggesting a role for Src kinases in the inhibition of chemotaxis following E-selectin treatment.

A



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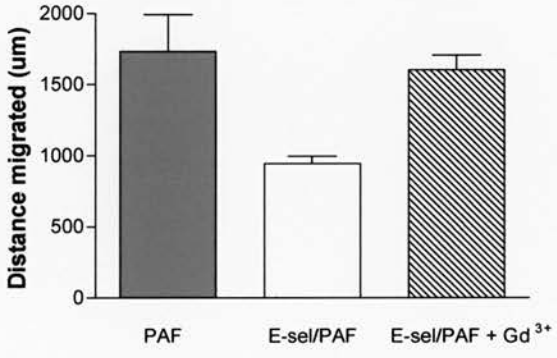
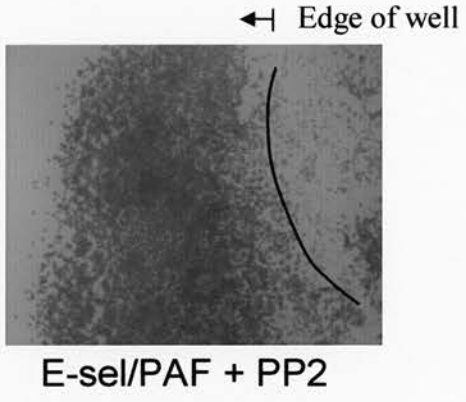


Figure 5.3 Calcium channel blockers reverse E-selectins inhibition

Chemotaxis of neutrophils was assessed using sub-agarose method (see section 2.9). (A) Images taken with a Zeiss Axiovert s100 with 10x objective. (B) After staining with Diff-Quick, the distance from the origin to the leading edge of migrating cells was determined using computer image analysis. Data shown are the mean $\pm$ sem from six separate experiments. E-selectin was used at 5µg/ml and Gd^{3+} used at 10µM.

A



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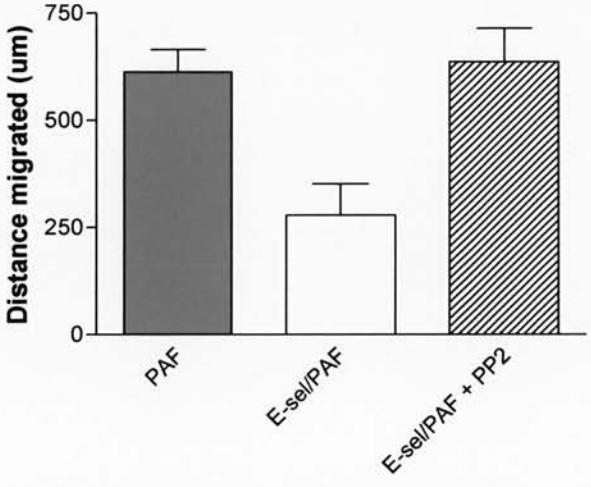

Figure 5.4 Src reverses E-selectin effects on chemotaxis

Chemotaxis of neutrophils was assessed using sub-agarose method (see section 2.9). Figure A shows images taken with a Zeiss Axiovert s100 with 10x objective. After staining with Diff-Quick, the distance from the origin to the leading edge of migrating cells was determined using computer image analysis (B). Data shown are the mean $\pm$ sem from four separate experiments. E-selectin was used at 5µg/ml and PP2 was used at a final concentration of 5µM.

Soluble E-selectin augments neutrophil respiratory burst

Increased $[Ca^{2+}]_i$ release following soluble E-selectin and PAF exposure may influence the potential for neutrophil-mediated cellular damage. Untreated neutrophils release low levels of O_2^- (Figure 5.5). However, treatment of neutrophils with E-selectin treatment alone significantly induced O_2^- release (Figure 5.5). E-selectin induced superoxide release was also blocked by ENA2, again indicating that the lectin domain of E-selectin was required for its functional effects. Analysis of the effects of E-selectin upon neutrophil superoxide response provides evidence that some of the observed effects of soluble E-selectin are independent of Ca^{2+} mobilisation. For example, E-selectin alone does not induce increased $[Ca^{2+}]_i$ but treatment of neutrophils with soluble E-selectin induces significant amounts of neutrophil O_2^- release (Figure 5.5) suggesting E-selectin induced prolongation of Ca^{2+} mobilisation in response to PAF is not involved in the effects of E-selectin on superoxide responses. In contrast, increased adhesion following E-selectin/PAF treatment may parallel prolonged Ca^{2+} mobilisation.

Soluble E-selectin induced SOCE is not involved in neutrophil priming

Classical priming agents such as PAF or TNF-α have no effect upon neutrophil superoxide release when used alone, but prime release in response to a second stimulus such as fMLP or chemokines. Untreated neutrophils release low levels of O_2^- and fMLP stimulation alone leads to a small increase in the level of O_2^- production, confirming that the neutrophils used in these experiments were minimally activated or unprimed (Figure 5.6). Treatment with PAF alone also did not induce neutrophil O_2^- release (untreated = 0.3 ± 0.1 nmoles $O_2^-/10^6$ cells and PAF alone = 1.3 ± 0.5 nmoles $O_2^-/10^6$ cells), but pre-incubation with PAF caused an increase from 3.0 ± 1.0 to 16.0 ± 2.0 nmoles $O_2^-/10^6$ cells in fMLP-induced O_2^- release (Figure 5.6).

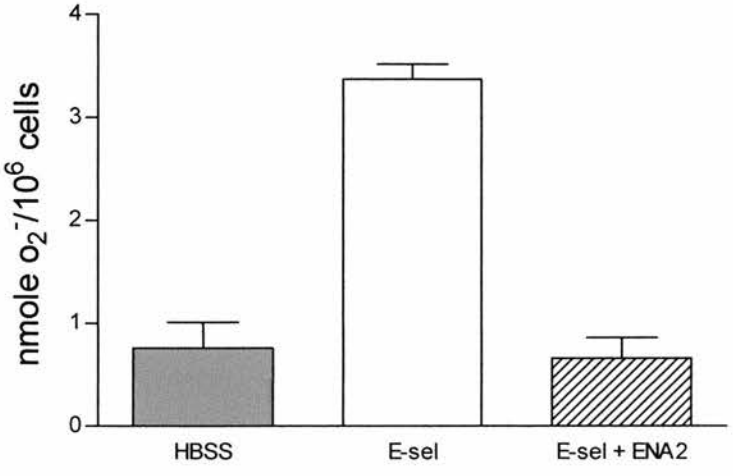

Figure 5.5 Soluble E-selectin alone induces O_2^- release

Neutrophils were preincubated with soluble E-selectin (5μg/ml) for 15 min at 37°C. For blockade of CD62E, ENA2 (1:50) and soluble E-selectin were preincubated for 1 h. Cells were then incubated in HBSS in presence of cytochrome c (1mg/ml) for another 15 min at 37°C. Superoxide dismutase-inhibitable reduction of cytochrome c was then determined by measuring the peak absorbance between 535 and 565nm using a Pye-Unican scanning spectrophotometer. Data are expressed as nmol of $O_2^-/10^6$ cells and are shown as the means ± sem of four separate experiments.

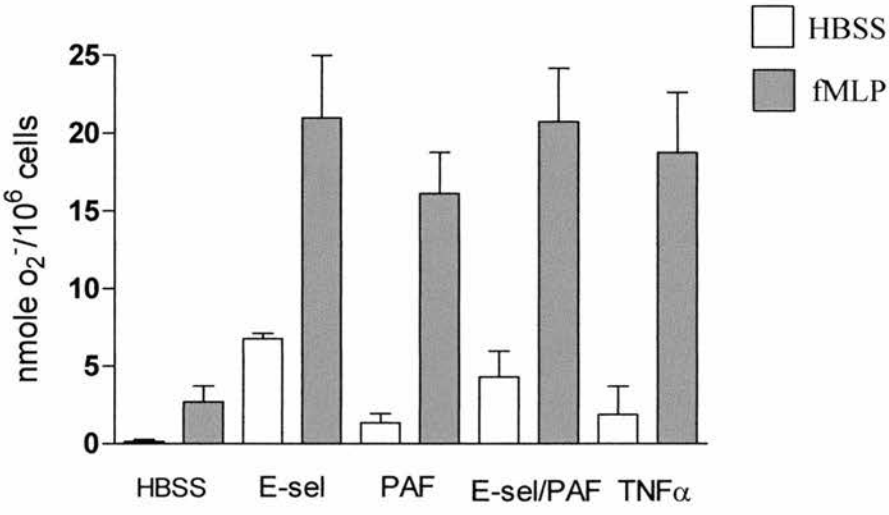

Figure 5.6 Soluble E-selectin effect upon O_2^- release involve distinct mechanism when compared to priming agents such as PAF and TNFα

Neutrophils were preincubated with soluble E-selectin (5μg/ml), PAF (100nM) for 15 min at 37°C or with TNFα (0.1ng/ml) for 1 h at 37°C. Cells were then incubated in HBSS of fMLP (100nM) in presence of cytochrome c (1mg/ml) for another 15 min at 37°C. Superoxide dismutase-inhibitable reduction of cytochrome c was then determined by measuring the peak absorbance between 535 and 565nm using a Pye-Unican scanning spectrophotometer. Data are expressed as nmol of $O_2^-/10^6$ cells and are shown as the means ± sem of four separate experiments.

In contrast, soluble E-selectin treatment alone significantly induced O_2^- release (4.0 ± 1.0 nmoles $O_2^-/10^6$ cells) and pre-incubation of neutrophils with soluble E-selectin prior to fMLP treatment resulted in an increase in O_2^- release to 20.0 ± 3.0 nmoles $O_2^-/10^6$ cells, to approximately the same levels as seen with PAF-primed neutrophils treated with fMLP (Figure 5.6). TNFα alone did not induce neutrophil O_2^- release but in combination with fMLP caused an O_2^- release of 19.0 ± 3.0 nmoles $O_2^-/10^6$ cells. Soluble E-selectin does not affect Ca^{2+} mobilisation in response to fMLP (Figure 3.5, Chapter 3). However, soluble E-selectin enhances fMLP's ability to act as a secretory stimulus in terms of O_2^- release. These data provide evidence that the effects of soluble E-selectin upon neutrophil superoxide production and release are largely independent of the effects upon Ca^{2+} mobilisation.

Soluble E-selectin augments PAF-induced neutrophil survival

The results in Chapter 3 (Figure 3.2), showed that soluble E-selectin prolongs PAF-induced Ca^{2+} mobilization. Recent investigations have suggested that Ca^{2+} has a critical regulatory role in neutrophil apopotosis and Whyte *et al.* (2003), demonstrated that transient elevation of $[Ca^{2+}]_i$ resulted in prolonged inhibition of neutrophil apoptosis. It has also been previously shown that neutrophil apoptosis characterized by distinctive alterations in nuclear morphology (Figure 5.7A) is associated with a reduction in E-selectin binding ability (Dransfield I, Stocks SC, 1995). These data suggest that the role of soluble E-selectin in neutrophil apoptosis is a pertinent mechanism to investigate. Therefore experiments investigating the effect of soluble E-selectin on apoptosis were carried out using surface expression of phosphatidylserine (PS) as an index of apoptosis. PS exposure is an indicator of apoptosis as following the onset of apoptosis, most mammalian cell types translocate PS from the inner face of the plasma membrane to the cell surface (Zhang G, 1997). Following 20 hours in culture, the viability of neutrophils was determined by flow cytometric analysis of annexin V binding and PI exclusion. Apoptotic neutrophils were defined as the population of cells, which were annexin V positive and PI negative and viable neutrophils are annexin V and PI negative.

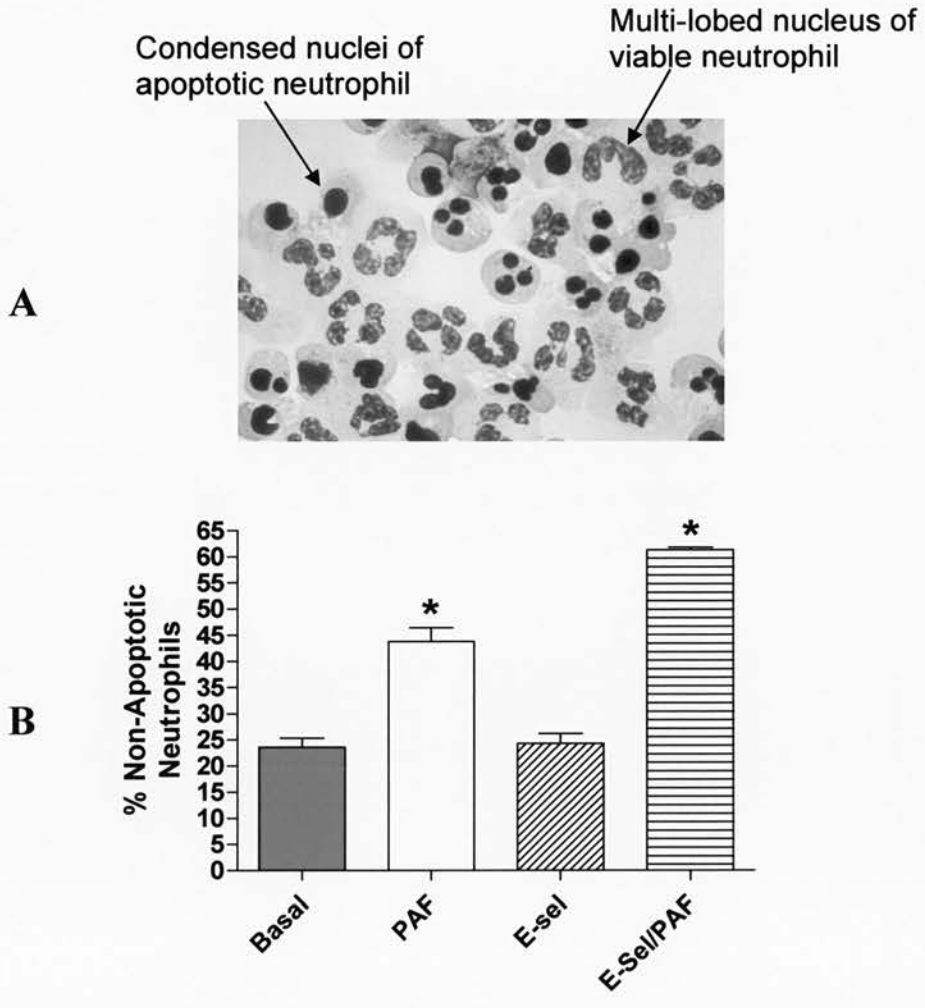

Figure 5.7 E-selectin augments PAF-induced survival

(A) In overnight cultured neutrophils, apoptotic nuclei appear condensed whilst viable cells have multi-lobed nuclei. (B) Viability was determined by flow cytometric analysis of annexin V binding and PI exclusion. Human neutrophils (5 x 10^6/ml) were cultured at 37°C in Iscove's DMEM containing 10% autologous serum and were treated with 100μM PAF and/or 5μg/ml soluble E-selectin. After 20 h, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine exposure. All values represent mean ± sem of n = 5 experiments, each performed in triplicate. * Indicated statistically different ($P<0.5$) from PAF treated controls.

Annexin V is a marker of apoptotic cells and PI binds to DNA and allows the detection of necrotic cells rather than apoptotic cells.

PAF has been shown by several groups to prolong neutrophil survival during *in vitro* culture (Kuijpers *et al.*, 2001; Khreiss *et al.*, 2004). Figure 5.7 shows that PAF causes a 2 fold increase in the percentage of non-apoptotic neutrophils present after 20 hours (42.8 ± 6.5 % vs 23.1 ± 4.2%) confirming that treatment with PAF alone promotes neutrophil survival. In contrast, treatment with soluble E-selectin alone does not affect neutrophil apoptosis. However, neutrophils cultured with both E-selectin and PAF, show a significant increase in neutrophil survival when compared with PAF alone. These data indicate that E-selectin augments PAF-induced neutrophil survival.

Role of E-selectin induced SOCE in PAF-mediated augmentation of apoptosis

To investigate if the effects of soluble E-selectin upon PAF-induced neutrophil survival were dependent or independent of E-selectin prolonged PAF induced Ca^{2+} mobilisation, the effect of Ca^{2+} channel inhibitors on the observed inhibition of apoptosis was investigated. Figure 5.8, shows that both MRS1845 and Gd^{3+}, known SOC channel blockers, inhibited E-selectin effects on PAF-induced neutrophil survival to levels observed in the presence of PAF alone. These results demonstrate that increased neutrophil survival observed in the presence of PAF requires E-selectin induced SOCE. Furthermore, the effects of soluble E-selectin upon neutrophil apoptosis may require a TRPC channel since Gd^{3+} also reverses the soluble E-selectin effect.

Role of signaling pathways in neutrophil apoptosis

The results in Chapter 4 (Figure 4.1) suggested a role for Src family tyrosine kinases and PI 3-kinase, in soluble E-selectin-induced SOCE. Therefore, experiments investigating the effect of several inhibitors on E-selectin and PAF apoptosis levels were carried out to identify roles for distinct signalling pathways.

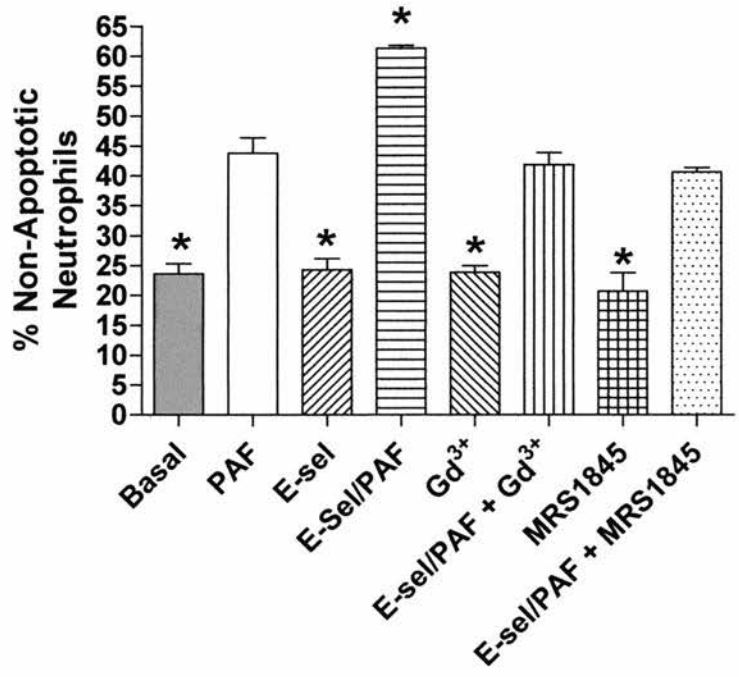

Figure 5.8 Effect of Ca^{2+} channel inhibitors on E-selectin augmentation of PAF-induced survival

Neutrophil viability was determined by flow cytometric analysis of annexin V binding and PI exclusion. Human neutrophils (5 x 10^6/ml) were cultured at 37°C in Iscove's DMEM containing 10% autologous serum and were treated with 1μM PAF and/or 5μg/ml E-selectin. For blockade with Ca^{2+} channel inhibitors 10μM Gd^{3+} or 2μM MRS 1845 was added. After 20 h, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine exposure. All values represent mean ± sem of n = 5 experiments, each performed in triplicate. * Indicated statistically different ($P<0.5$) from PAF treated controls.

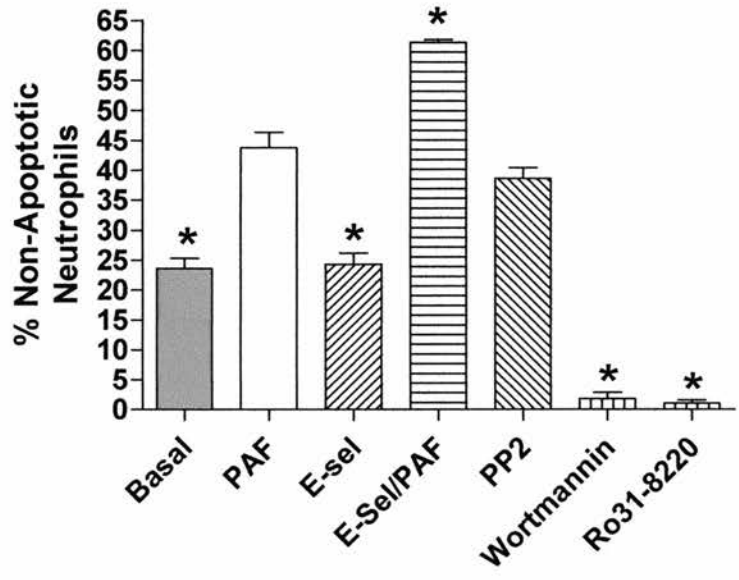

Figure 5.9 Effect of Src, PI 3-kinase and PKC on apoptosis levels

Neutrophil viability was determined by flow cytometric analysis of annexin V binding and PI exclusion. Human neutrophils (5 x 10^6/ml) were cultured at 37°C in Iscove's DMEM containing 10% autologous serum and were treated with 1μM PAF and/or 5μg/ml E-selectin. For blockade of Src, 5μM PP2 was added, for blockade of PI 3-kinase 100nM wortmannin and for blockade of PKC, 1μM Ro 31-8220 was added. After 20 h, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine exposure. All values represent mean $\pm$ sem of n = 5 experiments, each performed in triplicate. * Indicated statistically different ($P<0.5$) from PAF treated controls.

Treatment of neutrophils with PP2 (Src specific inhibitor), alone augmented neutrophil survival, whereas wortmannin (PI-3-kinase inhibitor), and Ro31-8220 (specific PKC inhibitor) alone induced apoptosis (Figure 5.9). Inhibition of these signalling pathways has direct effects upon neutrophil apoptosis and therefore it is difficult to interpret these results and due to time limitations further in-depth investigations were not carried out.

CD66 is a potential E-selectin counter-receptor

Previous data in this thesis demonstrates that the binding of soluble E-selectin via the lectin domain to a counter-receptor present on neutrophils is essential for the prolongation of Ca^{2+} mobilization in neutrophils in response to PAF. E-selectin is recognised by receptors that carry sialyl lewis^x, the principal carbohydrate counter-receptor for the selectins. One cell adhesion molecule suggested to play a role in selectin-mediated adhesion is CD66 or carcinoembryonic antigen (CEA). Human neutrophils are known to express; CEACAM1 (CD66a), CEACAM8 (CD66b), CEACAM6 (CD66c) and CEACAM3 (CD66d), all of which are highly glycosylated molecules with multiple sialyl and fucosyl residues (Skubitz *et al.*, 2001). As CD66 may function as an adhesion molecule capable of binding to E-selectin, experiments were carried out to examine the effect of CD66 ligation on PAF-induced Ca^{2+} mobilization. Pre-incubation of neutrophils with either a monoclonal antibody (Kat4c) or a CEA polyclonal antibody revealed that both antibodies prolonged PAF-induced induced Ca^{2+} mobilization in a similar manner to that caused by soluble E-selectin (Figure 5.10). These results suggest a possible involvement of CD66 in the PAF-induced $[Ca^{2+}]_i$ prolongation effect of soluble E-selectin.

PSGL-1 has been proposed as an important tethering counter-receptor for E-selectin and has been shown by several groups to support the initial tethering of leukocytes to E-selectin and is highly expressed on human leukocytes. Therefore, antibodies were used to investigate whether PSGL-1 was the neutrophil counter-receptor mediating soluble E-selectin PAF-induced Ca^{2+} mobilisation.

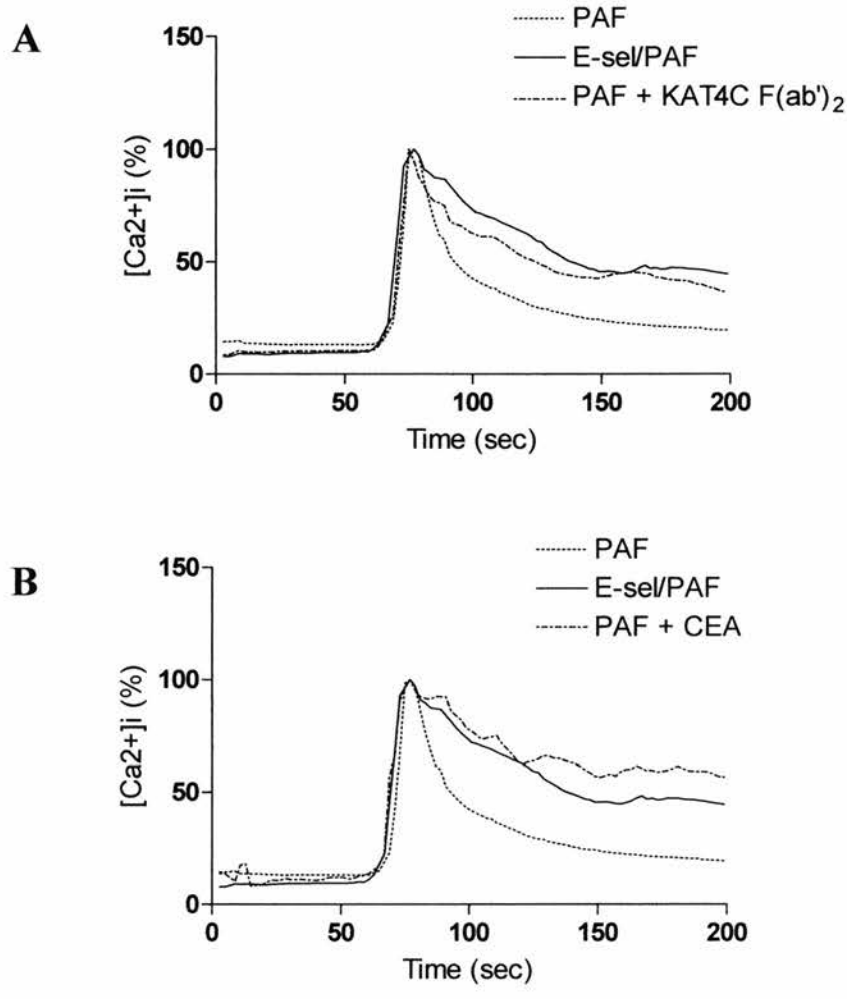

Figure 5.10 CD66 antibodies prolong PAF-induced Ca^{2+} mobilisation

Freshly isolated neutrophils were loaded with Fura-2AM (2μM) and pre-incubated with or without soluble E-selectin (5μg/ml) or CD66 antibodies (1:100), KAT4C F(ab')$_2$ (A) or CEA (B) for 15 min at 37°C. The cells were then stimulated with PAF (100nM) after 60 s of recording. A representative Ca^{2+} trace from three separate experiments that were performed.

Data from preliminary experiments showed that prolongation of Ca^{2+} mobilisation was inhibited by the function-blocking anti-PSGL-1 antibody (PL-1) whereas the non-blocking antibody (PL-2) was without effect, eliminating the possibility of steric effects of antibody binding to PSGL-1 (data not shown). Although, these results appeared to demonstrate a requirement for PSGL-1 in mediating the effects of soluble E-selectin upon neutrophil Ca^{2+} responses, subsequent experiments using different batches of antibodies from the same supplier failed to reproduce these findings. Therefore, the role of PSGL-1 in E-selectin mediated SOCE remains unresolved.

Identification of E-selectin counter-receptors

Several putative glycoprotein selectin counter-receptors have been isolated from hematopoietic cells using *in vitro* affinity purification techniques (Steegmaier *et al.*, 1995; Jones *et al.*, 1997; Dimitroff *et al.*, 2001), but the exact identity and contribution of physiologic E-selectin counter-receptors on neutrophils is unknown. Precipitation techniques were used to identify candidate glycoproteins as counter-receptors for E-selectin. Biotinylation of the neutrophil cell surface was carried out prior to affinity isolation of potential cell surface counter-receptors for E-selectin (Figure 5.11). IgG sepharose was then used to pull-down E-selectin or protein A from neutrophil lysates. As can be seen in figure 5.12 under the conditions used in these experiments E-selectin pull-downs failed to identify potential counter-receptors. An LFA-1 integrin mAb was used as a positive control to confirm that the cell surface biotinylation and precipitation protocol was successful and as can be seen in figure 5.12 the expected bands for LFA-1 were observed at 175 (α subunit) and 95kDa (β subunit). Protein A was used as an additional control to demonstrate specificity of any potential counter-receptors, as E-selectin contains a protein A domain which may bind to potential candidate proteins. This therefore, eliminates the possibility that proteins in the lysate are binding to the protein A domain. All precipitation samples were also western blotted with an anti-E-selectin antibody to confirm successful pull-down of E-selectin.

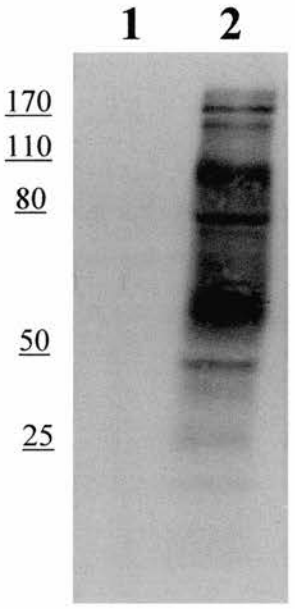

Figure 5.11 Biotinylation of the cell surface of neutrophils

Fresh neutrophils were incubated with 0.1mg/ml biotin and left for 1 h on ice and were then lysed as described in Materials and Methods (Section 2.12). Western blotting of unbiotinylated neutrophils (1) and biotinylated neutrophils (2) stained with streptavidin-HRP revealed numerous bands in the biotinylated neutrophil lane which proves biotinylation of the neutrophil cell surface has occurred. A representative immunoblot of three different experiments is shown.

Figure 5.12B shows that E-selectin was present in the E-selectin pull-down sample and none of the other precipitation samples as would be expected. Precipitation experiments can be difficult when using neutrophils, due to the potential for protein breakdown by the numerous proteases present in neutrophils. Furthermore, the E-selectin counter-receptor interaction is known to have fast association and dissociation rates, a preferred requirement for rolling adhesion, which may also make it technically difficult to detect by standard precipitation techniques. Any potential E-selectin counter-receptors may also be poorly biotinylated.

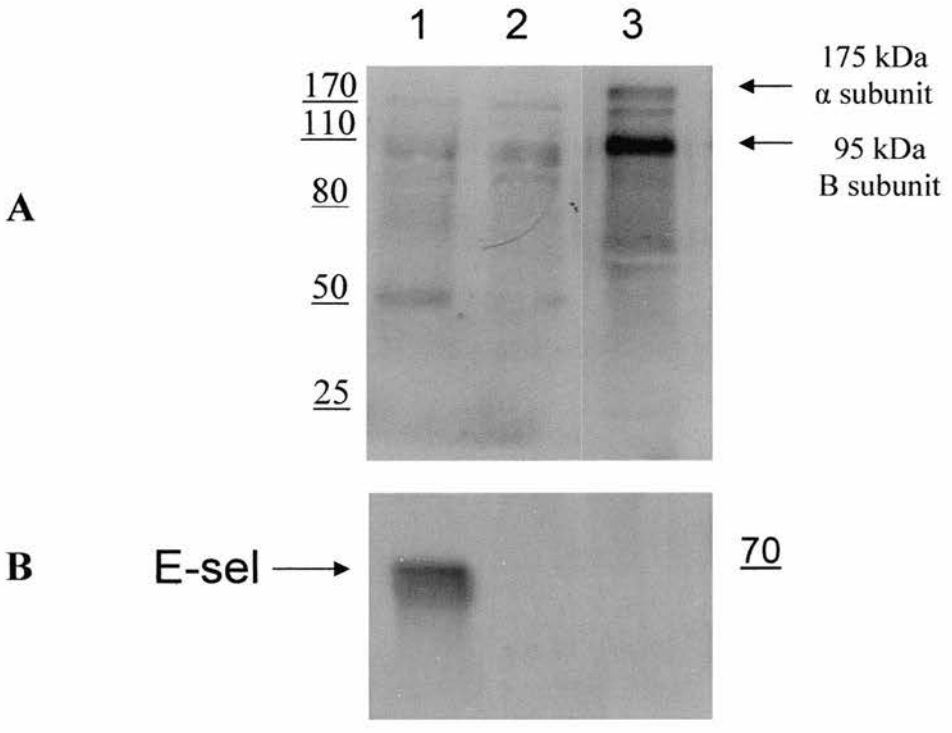

Figure 5.12 Precipitation of E-selectin counter-receptor

(A) Freshly isolated neutrophils were incubated with 0.1mg/ml biotin and left for 1 h on ice and were then preincubated with either 5µg/ml soluble E-selectin (lane1), 5µg/ml Protein A (lane2) or buffer (lane3) for 15 min prior and were then lysed as described in section 2.12. Cell lysates were then spun with the appropriate antibody for 1 h at 4°C (lane 1 & 2 no antibody was added & 2µg/ml of LFA1 was added in lane 3) before addition of the appropriate sepharose (LFA-1 – anti-mouse IgG sepharose and Protein A & E-selectin - rabbit IgG sepharose). In lane1 proteins that coprecipitated with soluble E-selectin were detected by probing the transfer membranes with an anti-streptavidin HRP. Lanes 2 & 3 were used as positive controls for precipitation, were also probed with anti-streptavidin HRP. (B) Pull-down of E-selectin was confirmed by probing the same samples with an anti-E-selectin antibody, 1:200. The results shown are representative of three independent experiments.

5.3 Discussion

Appropriate recruitment of neutrophils to sites of infection or tissue injury is critical for the initiation and progression of the inflammatory response. A number of studies have shown the importance of selectins in the orchestration of adhesion and migration of neutrophils in the initial capture and subsequent rolling on vascular endothelial ligands (Lawrence and Springer, 1991; Frenette *et al.*, 1996; Crockett-Torabi, 1998). Selectins also have a more complex regulatory role in inflammatory processes, initiating intracellular signal transduction cascades that lead to neutrophil activation and altered functional responses (Simon *et al.*, 1995; Hidari *et al.*, 1997). Many of the effector functions of neutrophils, such as granule release, phagocytosis and toxic metabolite production are modulated by the adhesive state of the cell. Engagement of β_2-integrins or matrix receptors serves to amplify the destructive potential of neutrophils in response to triggering stimuli (Graham *et al.*, 1993; Tyagi *et al.*, 2000a; Tyagi *et al.*, 2000b). Similarly, several studies have suggested that binding of neutrophils to vascular selectins may enhance subsequent neutrophil function (Kuijpers *et al.*, 1993; Stocks *et al.*, 1995). In Chapter 3, it was demonstrated that soluble E-selectin caused prolonged $[Ca^{2+}]_i$ release following stimulation with PAF. In this chapter, the physiological consequence of this prolonged $[Ca^{2+}]_i$ release was investigated on a number of important neutrophil functional responses.

Firstly, neutrophil adhesion was investigated as previous studies reported that soluble E-selectin specifically potentiates β_2-mediated adhesion (Ruchaud-Sparagano *et al.*, 2000). Experiments were carried out that confirmed soluble E-selectin alone was capable of supporting neutrophil adhesion (Figure 5.1). Treatment of neutrophils with both E-selectin and PAF induced a synergistic effect upon β_2-integrin mediated adhesion (Ruchaud-Sparagano *et al.*, 2000). Ruchaud-Sparagano *et al.* (2000) investigated whether E-selectin affected the PAF response by altering Ca^{2+} fluxes within neutrophils using different Ca^{2+} inhibitors. Their results showed that blocking the initial intracellular Ca^{2+} influx abolished E-selectin and PAF-mediated β_2-integrin activation

suggesting a role for Ca^{2+} influx in the regulation of neutrophil adhesion. However, Ca^{2+} mobilisation may not be involved in the regulation of neutrophil adhesion by E-selectin alone.

E-selectin's ability to augment β_2 integrin-mediated adhesion in response to PAF (Ruchaud-Sparagano *et al.*, 2000) suggested that soluble E-selectin effects on β_2 integrin-mediated directed migration of neutrophils may be an interesting mechanism to study. Therefore, experiments investigating the role of E-selectin upon neutrophil migration in response to PAF were carried out. The results showed that soluble E-selectin is a potent inhibitor of chemotaxis of neutrophils in response to PAF (Figure 5.2). The presence of tightly regulated local changes in $[Ca^{2+}]_i$ and transient increases of $[Ca^{2+}]_i$ in migrating neutrophils suggests that modulation of $[Ca^{2+}]_i$ may play a critical role in chemotaxis (Marks and Maxfield, 1990; Verploegen *et al.*, 2002). Therefore, Ca^{2+} influx mediated by E-selectin may be involved in inhibition of neutrophil migration by E-selectin. Figure 5.3 shows that MRS1845, a SOC inhibitor, reversed E-selectin effects on migration suggesting that Ca^{2+} influx may be involved. The prolonged elevation of $[Ca^{2+}]_i$ may interfere with the dynamic activation/deactivation of integrin ligand binding that are necessary for efficient migration (Lawson and Maxfield, 1995; Leitinger *et al.*, 2000). Figure 5.3 also shows Gd^{3+}, a TRPC inhibitor also reverses E-selectin's inhibitory effect on neutrophil migration implying that TRPC channels may be responsible for E-selectin's inhibitory effects.

One of the primary mechanisms by which neutrophils are activated is through the activation of protein tyrosine kinases at the plasma membrane (Berton, 1999; Fumagalli *et al.*, 2007). Among the tyrosine kinases, Src family kinases are activated upon stimulation of several cytokine and G-protein-coupled receptors in neutrophils (Yan *et al.*, 1995; Berton *et al.*, 2005) and therefore might play an important role in neutrophil signalling and effector functions. Data obtained using knock-out mice of different Src-family members demonstrate that these kinases are involved in degranulation (Mocsai *et al.*, 1999) and migration (Lowell and Berton, 1998). Src family tyrosine kinases are also

known to play a role in β_2 integrin-mediated adhesion (Giagulli *et al.*, 2006) and recently, Src family tyrosine kinases have been demonstrated to be involved in promoting β_2 integrin adhesiveness in neutrophils tethered by E-selectin (Totani *et al.*, 2006). Inhibitors of Src family tyrosine kinases may be useful in the treatment of several inflammatory diseases including ischemia-reperfusion injury, sepsis or acute lung injury due to the critical role that they play in the regulation of neutrophil activation and inflammatory responses (Korade-Mirnics and Corey, 2000; Okutani *et al.*, 2006).

Data in Chapter 3 showed that soluble E-selectin induced activation and tyrosine phosphorylation of Src and that Src was involved in E-selectin prolonged Ca^{2+} mobilisation, suggesting that the Src family tyrosine kinases may be important regulators in soluble E-selectin-mediated signalling in neutrophils. Therefore, the role that Src family kinases play in soluble E-selectin mediated signalling in chemotaxis was investigated. PP2, a Src family specific inhibitor, reversed soluble E-selectin effects on chemotaxis, returning chemotaxis to basal levels (Figure 5.4). These data indicate that Src family kinases play a role in the signalling events responsible for E-selectin inhibition of neutrophil chemotaxis. Kumar *et al.* (2001) showed soluble E-selectin mediated monocyte chemotaxis via Src family tyrosine kinases and soluble E-selectin-induced angiogenesis is predominantly mediated through the Src-PI3K pathway (Kumar *et al.*, 2003). Taken together, these results suggest that soluble E-selectin may inhibit chemotaxis through the Src pathway and this could thus be a potential target for modulating neutrophil or monocyte recruitment-driven diseases.

Figure 5.5 shows that soluble E-selectin significantly increases superoxide release when used alone, unlike conventional priming agents such as PAF or TNFα. However, in combination with stimuli that do not normally trigger O_2^- release from unprimed carefully prepared neutrophils (e.g. fMLP), soluble E-selectin potentiates O_2^- production. Previous work has shown that when interleukin-8 or PAF was used in combination with soluble E-selectin, the effect was the same as seen with fMLP (Ruchaud-Sparagano *et al.*, 2000). In Chapter 3, it was observed that soluble E-selectin specifically prolongs

Ca^{2+} mobilisation in response to PAF but not fMLP or LTB_4 whereas soluble E-selectin in combination with fMLP significantly induces production of O_2^-. Differential regulation of Ca^{2+} influx by fMLP and PAF has been well studied (Chen *et al.*, 2000). PAF and fMLP are also known to affect signalling pathways differently, for example neutrophil MAPK signalling pathways mediated by fMLP and PAF are known to be different (Chen *et al.*, 2005). This difference in signalling pathway activation may be the reason that PAF and fMLP produce different functional outcomes in neutrophils. The difference between augmentation of O_2^- and Ca^{2+} mobilisation in response to PAF indicates that altered Ca^{2+} mobilisation following PAF receptor ligation does not regulate the synergistic action of PAF and E-selectin upon O_2^- release.

Neutrophil death by disintegration or necrosis has the potential to inflict cellular damage by release of granule contents (Simon, 2003), stimulating resident phagocyte populations and prolonging the inflammatory reaction. While neutrophils undergo apoptosis constitutively, the rate of apoptosis can be modulated. For example it is known that inflammatory mediators, such as fMLP or C5a (Colotta *et al.*, 1992; Perianayagam *et al.*, 2002), as well as other receptor-mediated stimuli associated with Ca^{2+} mobilisation and elevation of $[Ca^{2+}]_i$ during neutrophil activation (Kuijpers *et al.*, 2001) can affect apoptosis. Elevation of $[Ca^{2+}]_i$ has been reported to induce apoptosis in a number of cell types (Jiang *et al.*, 1994; Chiu *et al.*, 2007; Sasaki *et al.*, 2007). However, a recent report showed that incubation of neutrophils with Ca^{2+} ionophores dramatically inhibited neutrophil apoptosis without inducing cell necrosis and concluded that transient elevation of $[Ca^{2+}]_i$ resulted in prolonged inhibition of neutrophil apoptosis (Whyte *et al.*, 1993). Therefore, these results raise the interesting possibility that apoptosis in the neutrophil is inhibited by stimuli increasing $[Ca^{2+}]_i$, whilst this event has been shown to trigger apoptosis in other cell types. It has also recently been demonstrated that Ca^{2+} influx is shutdown during apoptosis in neutrophils (Ayub and Hallett, 2004). This suggests that a failure of the shut-down mechanism could give rise to inappropriately prolonged activity, which may result in inflammatory tissue damage.

The results presented here showed that PAF significantly augmented neutrophil survival whereas soluble E-selectin alone failed to affect the rate of apoptosis at the time points examined. However, soluble E-selectin and PAF caused a subsequent significant prolongation of neutrophil survival when combined *in vitro*. To investigate if E-selectin's prolonged Ca^{2+} influx was involved in this process, the SOC inhibitors MRS1845 and Gd^{3+} were used. These SOC inhibitors did not affect basal levels of apoptosis but abolished the increased neutrophil survival observed following E-selectin and PAF co-treatment to levels observed with PAF alone, indicating that E-selectin-mediated SOCE may be involved in the augmented neutrophil survival. The ability of Gd^{3+}, a TRPC channel inhibitor, to reverse E-selectin's augmentation of PAF induced neutrophil survival implies there may be a role for TRPC channels in this response.

The data presented here suggests that transient rises in $[Ca^{2+}]_i$ can have profound and lasting effects upon on the apoptosis signalling pathway. PAF and E-selectin may be involved in inhibition of the pro-apoptotic signal in neutrophils. This study suggests a mechanism may exist for the inhibition of constitutive apoptosis, which is mediated, at least in part by elevation of $[Ca^{2+}]_i$. Apoptosis provides a neutrophil clearance mechanism in tissue and would tend to limit inflammatory tissue injury and promote resolution rather than persistence of inflammation. These observations may have some relevance for the development of novel approaches to anti-inflammatory therapy, as with increasing knowledge of the mechanism of apoptosis; it may be possible to specifically induce apoptosis in certain inflammatory cells at critical stages in the pathogenesis of inflammatory disease by inhibiting E-selectin and PAF's effects. It is therefore important to understand the signalling pathways involved in the Ca^{2+} influx during apoptosis in neutrophils before this knowledge may be useful in the future.

Investigation of the downstream signalling events involved in modulation of apoptosis was undertaken to identify roles for distinct pathways. The effects of several well characterised inhibitors on E-selectin and PAF modulation of apoptosis was determined. Treatment with PP2, the Src specific inhibitor alone augmented neutrophil survival

(Figure 5.9). In contrast the PI-3-kinase inhibitor wortmannin, and Ro31-8220, a specific PKC inhibitor alone induced apoptosis (Figure 5.9). Alteration of basal levels of apoptosis by these inhibitors makes investigation of the role of Src, PI 3-kinase and PKC in E-selectin's effect on PAF-induced apoptosis difficult.

Tyrosine kinases, PI-3-kinase and PKC isoforms have been shown by several groups to be important in inhibition of neutrophil apoptosis (Wei *et al.*, 1996; Webb *et al.*, 2000; Petrin *et al.*, 2006). PKC isoforms have been shown to be differently involved in regulation of apoptosis (Deacon *et al.*, 1997). For example PKCα has been demonstrated to be predominantly anti-apoptotic whereas PKCδ and PKCθ are pro-apoptotic (Pongracz *et al.*, 1999; Cataisson *et al.*, 2003). PI-3 kinase activity has also been shown to delay neutrophil apoptosis (Wang *et al.*, 2003). Unfortunately, in the experiments carried out here, inhibitors of these signalling pathways alone affected basal neutrophil apoptosis and did not allow definition of the role of signalling pathways involved in the effect of E-selectin on PAF-induced apoptosis. However, alternative approaches could be investigated to define the role of signalling pathways involved in the E-selectin regulation of PAF inhibition of apoptosis. For example to investigate the role of PKC signalling, specific PKC isoform inhibitors could be used to determine the role of each PKC isoform in neutrophil apoptosis and various concentrations of these inhibitors could be tested to find an optimal concentration that didn't affect basal apoptosis. The effect of PAF and E-selectin on triggered cell death by Fas of TNFα could also be tested, to see if they still promoted cell survival.

In Chapter 3 (Figure 3.9) TRPC channels were identified to be involved in the generation of soluble E-selectin induced SOCE. The results in this chapter demonstrate that TRPC channels may also be involved in mediating the Ca^{2+} fluxes that are responsible for the inhibitory effect of E-selectin on chemotaxis (Figure 5.3) and soluble E-selectin's augmentation of PAF induced neutrophil survival (Figure 5.8). However, neither of these experiments definitively identifies which TRPC channels regulate these responses. Therefore, to determine the role of TRPC in modulating neutrophil migratory

potential and neutrophil apoptosis, it would be interesting to carry out these functional assays using neutrophils isolated from wild type and TRPC6$^{-/-}$ mice. As discussed in Chapter 3, experiments to confirm that E-selectin and PAF cause permissive SOCE in neutrophils from mice, in the same way as human neutrophils would need to be carried out first. If neutrophils from TRPC6$^{-/-}$ mice treated with soluble E-selectin showed normal migration and no augmentation of PAF-induced neutrophil survival it would confirm the involvement of TRPC6 in these functional responses. It would also be interesting to investigate if failure to limit tyrosine kinase activation underlies generation of a 'pro-inflammatory' neutrophil response following selectin ligation resulting in altered chemotaxis, degranulation and survival by using murine neutrophils from Src kinase knockout mice. However, we still have to confirm if soluble E-selectin causes the same effects in murine neutrophils. As discussed in previous chapters, using murine neutrophils imposes constraints on the number of experiments that can be carried out as typical yields from bone marrow neutrophil and peritoneal neutrophil preparations are approximately 1-2 million cells in total.

Elevated levels of soluble E-selectin are found in patients with inflammatory conditions including septic shock (Kayal *et al.*, 1998), rheumatoid arthritis (Koch *et al.*, 1993; Yildirim *et al.*, 2005), juvenile idiopathic arthritis (Bloom *et al.*, 2005), ARDS (acute respiratory distress syndrome) (Ruchaud-Sparagano *et al.*, 1998), hepatitis B (Yan et *al.*, 2006), asthma (Janson *et al.*, 2005) and in uveitis (Lee *et al.*, 2007). However, the consequences of elevated levels of soluble E-selectin at inflamed sites and the mechanism of action of soluble E-selectin are still poorly understood. The results in this chapter suggest that the presence of high local concentrations of soluble E-selectin would have the potential to influence cellular recruitment patterns and may exert pro-inflammatory effects upon neutrophil behaviour. Soluble E-selectin specifically augmented β_2 integrin-mediated adhesion decreased PAF-directed migration of neutrophils and triggered O_2^- release, which would result in tissue damage *in vivo*. Alterations in Ca^{2+} mobilisation in response to soluble E-selectin and PAF synergistically augment neutrophil survival, which could lead to prolonged survival of

destructive neutrophils at sites of inflammation. These results may suggest that soluble E-selectin may act to prolong neutrophil-endothelial contact and trigger oxidant-mediated cellular damage. They also imply that there is potential to selectively block some of soluble E-effects by inhibiting Ca^{2+} influx, leaving other Ca^{2+} mediated responses intact, which may allow some host defence functions to be maintained and could be very useful clinically.

The lectin domain of E-selectin plays a major role in counter-receptor recognition and binds to sialyl Le^x on neutrophils. Importantly, we have shown a requirement for the lectin-binding domain in several E-selectin mediated functional responses including adhesion, chemotaxis and superoxide production. It is also required for the prolongation of Ca^{2+} mobilization in neutrophils in response to PAF. Therefore, identification of the neutrophil counter-receptors for E-selectin is a critical issue, as they could represent useful pharmacological targets for inflammatory diseases. There are a number of potential counter-receptors for E-selectin present on neutrophils, including PSGL-1, CD44 and a number of proteins that express sialyl Le^x, including CD66.

E-selectin was shown to require a lectin-binding domain for several of its functional responses and CD66 is known to bind to lectin-binding domains. In neutrophils CD66a is the major membrane glycoprotein that binds sLe^x antibodies (Stocks and Kerr, 1993). Thus CD66a may be an endogenous ligand for C-type lectins, such as E-selectin. Recently, several different Lewis x glycan epitopes were identified on CD66a that may function as specific binding structures for other proteins, such as C-type lectins using MALDI-TOF MS analysis (Lucka *et al.*, 2005). CD66 family members also contain a sequence similar to immunoreceptor tyrosine-based activation motifs (ITAM) and these motifs are known to be involved in activation of leukocyte responses. CD66a and CD66d have been shown to contain one ITAM motif in their cytoplasmic domain (Nair and Zingde, 2001; McCaw *et al.*, 2003). CD66a contains a YstL sequence and CD66d contains a YeeL sequence. The tyrosine residues within the ITAM motif are necessary and sufficient for ITAM signalling activity and, when phosphorylated, bind to SH2

domains of Src kinases (e.g. Fyn, Lck) and of other tyrosine kinases (e.g. Syk and ZAP-70) which can phosphorylate recruited adaptor proteins (Pitcher and van Oers, 2003). It has recently been shown that integrin signalling in neutrophils and macrophages requires adaptors containing ITAM motifs (Mocsai *et al.*, 2006). Therefore, one possibility is that the presence of an ITAM-like sequence in the CD66 cytoplasmic domain may provide a mechanism for regulation of neutrophil function following E-selectin binding.

The data presented here strongly implicates CD66 in the response to E-selectin, as CD66 ligation with antibodies caused prolonged PAF-induced Ca^{2+} mobilization in a similar manner to that caused by soluble E-selectin. It would be critical to demonstrate that CD66 functions as a receptor for E-selectin on neutrophils. Immunofluorescence or immunoprecipitation techniques could be used to show association of E-selectin with membrane CD66. To date, preliminary experiments with biotinylated neutrophil surface receptors and immunoprecipitating potential counter-receptors were unsuccessful, probably due to the high dissociation rates between E-selectin and it's ligands. It would have been interesting to use an unbiased cross-linking approach to identify neutrophil surface receptors that bind to soluble E-selectin. Neutrophil surface receptors would be biotinylated and reversible cross linkers such as 3,3′-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) or disuccinimidyl suberate (DSS) could be used to cross link closely associated molecules. Specific immunoprecipitation of E-selectin followed by reversal of cross-linking would allow the identification of neutrophil molecules that bind E-selectin by SDS-PAGE together with immunoblotting. As a control, P-selectin could be used as it binds to neutrophils but fails to affect neutrophil functional responses examined here. Direct binding of recombinant purified CD66 to the selectins used in this study could also be tested using surface plasmon resonance techniques. Experiments using monoclonal antibodies could be used to selectively engage specific CD66 family members, and define their contribution to prolongation of Ca^{2+} and augmentation of neutrophil functional responses. A number of proteins would be anticipated to associate with E-selectin, binding either independently or as a complex. Examination of the binding of E-selectin and co-localisation of

molecules identified as E-selectin counter-receptors using confocal microscopy might also provide additional insight into whether receptor complexes are involved.

If CD66 was an E-selectin counter-receptor the ITAM-like sequences, YstL or YeeL, present in the CD66 cytoplasmic domains may recruit Fyn or Lck and these may target the TRPC channel directly. By using a series of fusion proteins with mutations in key tyrosine residues, it would be possible to determine the contribution of specific regions of the CD66 cytoplasmic domain in the regulation of TRPC activity.

Interestingly, a recent study has shown that G-CSF directly induces E-selectin expression on human myeloid cells (Dagia *et al.*, 2006). They found that G-CSF treatment consistently resulted in up-regulation in expression of HCELL (a specialized sialofucosylated glycoform of CD44) and PSGL-1 and suggested that these ligands prime neutrophils to adhere to inflamed endothelium. On the basis of these results it would be interesting to investigate the effect of G-CSF treated neutrophils in immunoprecipitation experiments to investigate if HCELL is a candidate as an E-selectin counter-receptor. A cell line which expresses the components required for this system to work, for example the PAF receptor, TRPC6 and CD66, could be used to determine if CD66 was an E-selectin counter-receptor or used to identify potential E-selectin counter-receptors.

The results in this chapter have shown that binding of E-selectin via the lectin domain, through an as yet unidentified E-selectin counter-receptor present on neutrophils, is required for E-selectin to mediate several of its functional responses. The data presented in this study suggest that CD66 antigens may be involved in events that regulate E-selectin-mediated prolongation of PAF-induced $[Ca^{2+}]_i$. One possibility is that CD66 antigens contain a binding site for soluble E-selectin or CD66 and other adhesion receptors need to co-engage via soluble E-selectin to regulate Ca^{2+} signalling in response to PAF.

Exposure of neutrophils to soluble E-selectin alters the capacity for production of damaging reactive oxygen species in a manner that is distinct from classical priming agents but is not dependent on the alteration of Ca^{2+} mobilisation seen in response to E-selectin and PAF. Neutrophil β_2-integrin-mediated chemotaxis is significantly inhibited following exposure to E-selectin and E-selectin augments PAF-induced neutrophil survival. These results demonstrate a requirement for E-selectin induced SOCE and TRPC channels therefore suggest that E-selectin mediated Ca^{2+} mobilisation may affect neutrophil apoptosis and migration. In conclusion, soluble E-selectin levels are elevated in many chronic inflammatory conditions and soluble E-selectin promotes neutrophil destructive function, which could influence progression of inflammation. Therefore understanding mechanisms by which selectins influence signalling pathways may reveal a novel therapeutic target for the treatment of inflammatory disease.

Chapter 6 - Summary & Future Directions

6.1 Summary

The main aim of this thesis was to study the effects of soluble E-selectin on Ca^{2+} mobilization in neutrophils, and the signalling events involved in its regulation. The principal finding of this thesis was that soluble E-selectin does not mobilize $[Ca^{2+}]_i$ directly, but that signalling via E-selectin receptors allows molecular crosstalk with receptors for specific inflammatory mediators (e.g. PAF) to occur, permitting a novel form of Ca^{2+} influx through TRPC channels and modulation of neutrophil responses. I have demonstrated that soluble E-selectin induced modulation of TRPC channel activity occurs through phosphorylation and activation of Src-family tyrosine kinases in neutrophils, via activation of PI 3-kinase. I have also shown a potential role for PKC in regulating TRPC6 channel activity. This study provides the first evidence for intracellular communication between receptors for E-selectin and $G_{q/11}$-coupled inflammatory mediator receptors and suggests this response is mediated through regulation of TRPC channels (Figure 6.1). Another important finding was that E-selectin triggered alterations in TRPC channel function acts to promote adhesion, reduce migratory capacity and augment neutrophil destructive potential and neutrophil survival. Preliminary data also suggested that CD66 has the potential to act as a counter-receptor for E-selectin and mediate similar neutrophil responses.

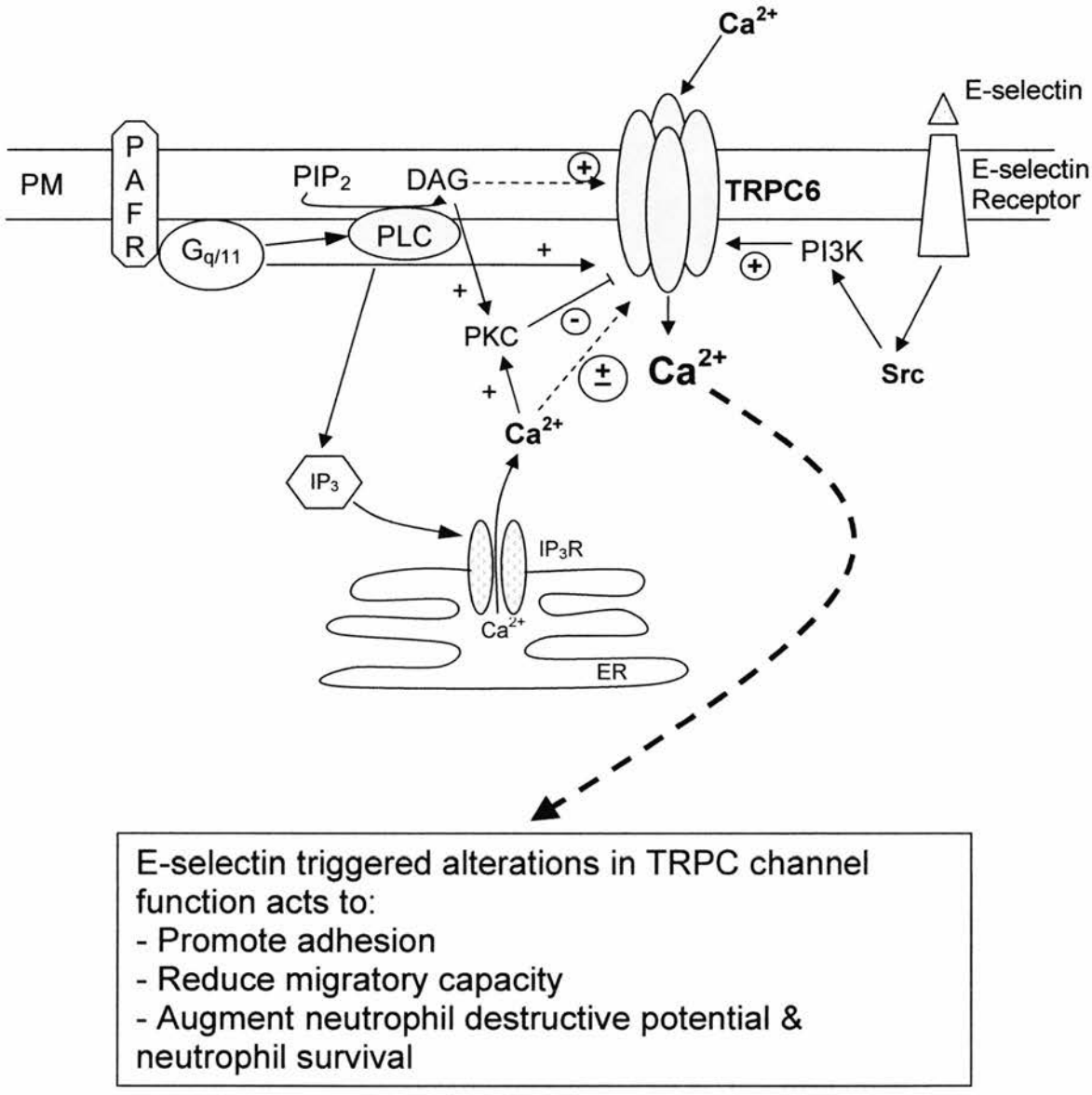

Figure 6.1 - A novel mechanism of molecular cross-talk between an adhesion receptor and an inflammatory mediator.

The results presented in this thesis predict the above model for intracellular communication between an adhesion receptor and inflammatory mediator at a molecular level, through activation of permissive SOCE by selectin receptor ligation following PAF stimulation of human neutrophils and present the functional outcomes of this process.

6.2 Why is this work important?

Inappropriate recruitment and adhesion-dependent degranulation of neutrophils contributes to the progression of several inflammation diseases including rheumatoid arthritis, ARDS, COPD and asthma (Stockley, 1999; Puneet *et al.*, 2005; Eyles *et al.*, 2006). Soluble E-selectin levels are elevated in many chronic inflammatory conditions and soluble E-selectin has been suggested to promote neutrophil destructive function which could influence progression of inflammation. Inhibition of neutrophil 'rolling' adhesion by blocking selectins has been shown to affect the accumulation of neutrophils in many experimental settings (Xia *et al.*, 2002; Mocco *et al.*, 2002) and consequently the possibility that inhibiting selectin activity might provide a treatment for inflammatory disorders has attracted much attention. However, pre-clinical trials and clinical trials using drugs that block selectin activity has proven disappointing as inhibiting adhesion receptors in neutrophils can lead to the loss of vital anti-microbial defences. Whilst therapeutic blockade of adhesion receptors may place patients at risk of life-threatening infections due to inadequate recruitment of inflammatory cells, selective targeting of adhesion receptor signalling pathways could be exploited to attenuate the destructive responses of neutrophils. Thus, understanding the mechanisms by which selectins influence signalling pathways may be very important in the discovery of novel drug compounds for inflammatory diseases.

In particular, Ca^{2+} is known to play a central role in the functional activities of neutrophils that are important for host defence (Nathan, 2006). Recent exciting reports have shed new light on the components and regulators of store-operated Ca^{2+} entry (Liou *et al.*, 2005; Spassova *et al.*, 2006; Peinelt *et al.*, 2006) and disruption of their underlying control mechanisms may lead to inappropriate cell activation, contributing to development of major life threatening diseases including atherosclerosis, rheumatoid arthritis, COPD and allergic reactions (Tian *et al.*, 2004; Steel and Anderson, 2004). The work presented in this thesis aims to define the mechanisms involved in Ca^{2+} signalling,

which may ultimately allow specific uncoupling of neutrophil function to limit their impact on disease progression.

There are over 300 ion channels identified in humans and defects in ion channel function are widely suspected to be the cause of various diseases (Vincent *et al.*, 2006; Nilius *et al.*, 2007). Abnormal regulation of ion channel function is especially interesting in all forms of inflammation and in systemic diseases, such as neurodegenerative, cardiovascular, and respiratory diseases. However, only a few ion channels have been directly linked to human disease and less than 10% of known Ca^{2+} channels have been successfully used as therapeutic targets. In particular, TRP channels are often activated by multiple and diverse gating stimuli and act as molecular integrators of these external and/or internal signals. Indeed, five diseases associated with defects in genes encoding the TRP channels have been identified to date (Nilius *et al.*, 2007). Perturbation of physiological functions mediated by ion channels can also contribute more subtly to the genesis of disease as a consequence of changes in channel abundance, or channel sensitization, or desensitization, resulting in exaggerated, or diminished, responses to important pathological stimuli.

The TRPC6 gene has been linked to hereditary kidney disorder, focal & segmental glomerulosclerosis (FSGS), which leads to renal failure. Six families with six different TRPC6 gene mutations have been identified (Winn *et al.*, 2005; Reiser *et al.*, 2005). These mutations lead to a kidney disease with late onset and a variable rate of progression to FSGS. Three of the identified mutations of the TRPC6 gene are missense mutations that result in enhanced signalling (gain of function) by TRPC6 via specific amino acid substitutions. In the nephron, TRPC6 is expressed in the podocytes in the kidney glomerular filter (Winn *et al.*, 2005; Reiser *et al.*, 2005). Podocyte foot processes and the glomerular slit diaphragm form the glomerular filter and are an essential part of the permeability barrier in the kidney, which is defective in FSGS (Kriz, 2005; Durvasula and Shankland, 2006). The defect in the filter function results in proteinuria and end-stage renal failure (Kriz, 2005; Gudermann, 2005). It is uncertain how the

mutations in TRPC6 translate into the development of FSGS. However, for the mutations that result in gain of function, enhanced Ca^{2+} entry may constitute a pathogenic trigger, such as a Ca^{2+} overload of the podocyte that initiates cell death by apoptosis, or causes dysregulation that compromises the integrity of the permeability barrier (Reiser *et al.*, 2005). Alternatively, mutations in TRPC6 may impair the ability of the podocyte to adapt to the normal physiological demands of maintaining the functional glomerular filter, such as responding to changes in glomerular filtration pressure.

The presence of several TRPC genes and proteins in airway smooth muscle cells products has also been demonstrated, for example TRPC1 mRNA in rat bronchial smooth muscle cells (Sweeney *et al.*, 2002) and TRPC4 and TRPC6 mRNA in myocytes isolated from human trachea (Corteling *et al.*, 2004). The potential role of TRPC6 in airway smooth muscle cells has been identified from studies in vascular smooth muscle where RNA-knockdown of endogenous TRPC6 protein expression reduced spasmogen-activated increases in $[Ca^{2+}]_i$ and myogenic tone (Inoue *et al.*, 2001; Welsh *et al.*, 2002). Increased TRPC6 expression has been reported in patients with idiopathic pulmonary hypertension (IPAH) (Yu *et al.*, 2004). Inhibition of endogenous TRPC6 expression by using siRNA markedly attenuated IPAH-pulmonary artery smooth muscle cell proliferation, which suggests that overexpression of TRPC6 in pulmonary artery smooth muscle cell may play an important role in the development of pulmonary medial hypertrophy (Yu *et al.*, 2004). TRPC6 has also been demonstrated as a key regulator of hypoxic pulmonary vasoconstriction (HPV), which occurs in ventilatory disorders including chronic pulmonary hypertension (Weissmann *et al.*, 2006). The TRPC channels have also been suggested to be involved in the development of many neurological diseases and a recent study suggested a role for TRPC6 in Alzheimer's disease (Lessard *et al.*, 2005). TRPC channels have also been shown to be involved in numerous other diseases which affect the cardiovascular, endocrine, gastrointestinal and reproductive systems (Nilius *et al.*, 2007).

In light of the data presented in this thesis relating to the role of TRPC6 in regulation of inflammatory cell functions, investigating the mechanism of inter-receptor communication that results in altered gating of TRPC channels in neutrophils could be very important for the development of anti-inflammatory drugs to attenuate leukocyte activation. Selective modulation of distinct components of these Ca^{2+} signals may be a potential target. Unfortunately, our knowledge of the detailed mechanisms through which TRP channels function is still elementary. Such a situation impairs both our understanding of the mechanistic role of TRP channels in human disease and the development of drugs to target TRPs and their specific functions. The more we learn about fundamental TRP channel physiology and the potential role of TRPs in disease, the closer we come to the development of novel therapies.

6.3 Future studies

A key focus of any future studies would be to define the molecular mechanism of communication between E-selectin receptors and TRPC channels responsible for augmenting neutrophil responses. The development of a model system would be instrumental in further unravelling this molecular mechanism of communication between an adhesion receptor and a Ca^{2+} channel. A cell line which expresses the components required for this system to work, for example the PAF receptor, TRPC6 and potential E-selectin counter receptors would be required. A cell line with these properties could also be used to determine if CD66 was an E-selectin counter-receptor or used to identify other potential E-selectin counter-receptors such as PSGL-1 or CD44.

Preliminary screening of various human leukemic cell lines e.g. THP-1, HL-60, U937 cell lines suggests that a cell line with expression of the required receptors is not available but it would be possible to develop a model system by transfecting in the required components into a cell line such as Rat basophilic leukemia cells (RBL-2H3), which have been used extensively as a model cell system for chemoattractant receptor signalling. RBL-2H3 cells are also a well established model for Ca^{2+} signalling as they

have been used in CRAC and SOC channel studies (Liu *et al.*, 2004; Csutora *et al.*, 2006; Tani *et al.*, 2007). The presence of TRPC6 has also been demonstrated in RBL-2H3 cells (Garcia and Schilling, 1997). RBL-2H3 cells contain small secretory granules, the majority of which stain with alcian blue and lack electron-dense material (Seldin *et al.*, 1985). These granules, however, reproduce the secretory process, which occurs in basophils and mast cells when stimulated through an IgE receptor-mediated mechanism or by a variety of non-immunological stimuli. IgE-mediated degranulation is an energy-requiring secretory event that occurs in the absence of cell lysis and depends on a number of signalling pathways resulting in a rise of cytosolic calcium ions (Beaven and Metzger, 1993). RBL-2H3 cells are also easily transfected and can be induced to degranulate as a readout for cell activation making them ideal as a model system for this project. Establishment of a model system also has the advantage that individual signalling pathways can be investigated more systematically by either studying the effect of knocking out specific components in the cell line or changing the combination of molecules that are transfected into the cell line.

Other important work that should be carried out to extend the studies reported here would be to investigate the role of TRPC channels in the processes of neutrophil adhesion, migration and release of neutrophil enzymes and reactive oxygen species. Established assays could be used for neutrophil adhesion, apoptosis, degranulation, superoxide generation and a combination of sub-agarose, transwell and trans-endothelial assays to determine the role of TRPC in modulating neutrophil migratory potential using neutrophils isolated from wild type and TRPC6$^{-/-}$ mice. In Chapter 5, I demonstrated that soluble E-selectin can augment neutrophil survival induced by the inflammatory mediator PAF (Figure 5.7). As un-regulated prolonged survival of destructive neutrophils at sites of inflammation will exacerbate tissue damage, the onset and rates of neutrophil apoptosis could be examined by flow cytometry from control and TRPC6$^{-/-}$ mice, to establish the potential of targeting this as an effective treatment to reduce neutrophil recruitment and survival at these inflamed sites.

Further functional characterizations of the signalling pathways that regulate TRPC are required. The results presented here indicate that multiple signalling events regulate TRPC activity in neutrophils. E-selectin was shown to induce activation of Src in neutrophils and E-selectin was also proven to alter the gating of TRPC channels. It would be interesting to determine whether tyrosine phosphorylation and altered protein-protein interactions acts to regulate TRPC channel activity in neutrophils. Immunoprecipitation and western blotting techniques combined with the use of specific inihibitors of individual components of these pathways could be used to determine the role of the Src/PI 3-kinase pathway in phosphorylation of TRPC6. Neutrophils from $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice (Totani *et al.*, 2006) could be used to investigate any potential molecular crosstalk between Src family kinases and TRPC6 channels. The role of PKC in TRPC phosphorylation also needs to be studied in further detail. Future experiments could be carried out to determine whether there are distinct sites for phosphorylation that can modulate TRPC6 channel opening, one of which (PKC targeted) that causes negative regulation and another (Src and/or Akt targeted) that modulates the channel in a positive manner. Immunoprecipitation and phosphopeptide analysis together with MALDI/TOF mass spectrometry could be used to identify and confirm roles for distinct phosphorylation sites of TRPC6 in neutrophils. A key aspect of these studies would be to determine the interplay between Src/PI 3-kinase pathway and PKC in control of neutrophil adhesion, migration and degranulation responses to soluble E-selectin and thus the potential for therapeutic modulation of neutrophil destructive potential.

STIM1 and CRACM1 have recently been identified as regulators of SOCE. Therefore, a key area of interest would be to investigate the role of STIM1 and CRACM1 in E-selectin regulation of TRPC channels. Data in Chapter 3 showed that neutrophils contain mRNA for STIM1 and CRACM1. Recent reports suggest that adaptor proteins recruiting other signalling molecules could promote communication to occur through protein/protein interactions. Formation of STIM1 homo-aggregates may be expected to appear as distinct punctuate regions close to the plasma membrane and by using single cell imaging with real-time confocal microscopy the localisation of STIM1, CRACM1,

E-selectin receptors and TRPC6 channels following E-selectin receptor ligation in the presence or absence of PAF could be determined. Together these studies would allow identification of protein/protein interactions with TRPC6 following stimulation of neutrophils with E-selectin and PAF. Huang *et al.* (2006) have recently shown that STIM1 co-immunoprecipitates with TRPC1, TRPC2 and TRPC4 but not with TRPC3, TRPC6 or TRPC7. This suggests that STIM1 is a key regulator of activity rather than a channel component. Therefore, I would expect phosphorylation events to underlie the 'priming' of TRPC6 that occurs, allowing it to interact with a $G_{q/11}$-derived signal that generates interaction sites on the channel itself or recruitment of adaptor proteins for STIM1 or other Ca^{2+} sensors.

Another key focus of any future studies would be to investigate the role of G_q in communication with TRPC channels. Previous results demonstrated that E-selectin permitted Ca^{2+} influx to occur in response to PAF but not fMLP of LTB_4 (Ruchaud-Sparagano *et al.*, 2000). Experiments in Chapter 3 where neutrophils were pre-treated with pertussis toxin identified that the PAF receptor was coupled to PLC through a PTX-insensitive $G_{q/11}$ pathway. This observation may, at least in part, explain the specificity of the observed effect of soluble E-selectin as fMLP and LTB_4 receptors are coupled through PTX-sensitive G proteins. Direct phosphorylation of TRPC6 alone appears to be insufficient to cause channel opening and Ca^{2+} influx and that a $G_{q/11}$-derived signal is also an essential requirement to permit the channel to open following Ca^{2+} store depletion. Thus, E-selectin receptor ligation may 'prime' the TRPC channels for a specific G protein signal which is supplied following stimulation of neutrophils with PAF at sites of tissue injury and inflammation. Therefore, it would be interesting to examine the association of $\alpha_{q/11}$ or $\beta\gamma$ subunits with TRPC6 by cross-linking and immunoblotting techniques and by using specific peptide inhibitors such as GP antagonist 2A (inhibits G_q) and mSIRK ($\beta\gamma$ subunit binding peptide) in reversibly-permeabilised neutrophils loaded with Fura2-AM and under whole cell patch clamp conditions to ascertain the role of G protein subunits in initiating TRPC6 channel opening.

In summary, the work in this thesis revealed a novel communication between an adhesion receptor and an inflammatory mediator receptor that could provide new opportunities for modulation of neutrophil function.

Chapter 7 - References

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E-selectin permits communication between PAF receptors and TRPC channels in human neutrophils

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The selectin family of molecules (L-, P-, and E-selectin) mediates adhesive interactions between leukocytes and endothelial cells required for recruitment of leukocytes to inflammatory sites. Soluble E-selectin levels are elevated in inflammatory diseases and act to promote neutrophil β_2-integrin–mediated adhesion by prolonging Ca^{2+} mobilization. Although soluble E-selectin alone was unable to initiate Ca^{2+} signaling, it allowed a novel "permissive" store-operative calcium entry (SOCE) following the initial platelet-activating factor (PAF)–induced release of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)–sensitive stores. This induction of permissive SOCE in response to soluble E-selectin and PAF was shown to act through a G protein-coupled receptor (GPCR) coupled to pertussis toxin-insensitive $G_{q/11}$. Furthermore, we demonstrated that permissive SOCE was mediated by canonical transient receptor potential channel (TRPC) due to its sensitivity to specific inhibition by MRS1845 and Gd^{3+} and that TRPC6 was the principal TRPC family member expressed by human neutrophils. In terms of mechanism, we demonstrated that soluble E-selectin activated Src family tyrosine kinases, an effect that was upstream of phosphatidylinositol 3′-kinase in a signaling pathway that regulates permissive SOCE following exposure of neutrophils to PAF. In summary, this report provides the first evidence for communication between an inflammatory mediator and adhesion receptors at a molecular level, through selectin receptor ligation allowing permissive SOCE to occur following PAF stimulation of human neutrophils. (Blood. 2006;107:4938-4945)

Introduction

Dysregulation of neutrophil granulocyte function has been implicated as having a key role in the initiation and progression of a number of inflammatory diseases. The control of events involved in leukocyte recruitment is critical for development of effective antimicrobial defenses and also for efficient wound healing. However, excessive inflammatory-cell recruitment or inappropriate cell activation leads to the development of chronic inflammation that favors fibrotic repair and, ultimately, loss of organ function. The selectin family of molecules (L-, P-, and E-selectin) mediate adhesive interactions between leukocytes and endothelial cells, representing one of the earliest events in the recruitment of inflammatory cells. Studies in vitro and in vivo have revealed the critical role of the selectin family of molecules in the initial capture and subsequent rolling adhesion on vascular endothelial ligands[1] before neutrophils firmly adhere and undergo diapedesis at sites of tissue injury and inflammation. In terms of structure, the selectins are type I transmembrane receptors that contain an amino terminal Ca^{2+}-dependent (C-type) lectin domain that has been shown to be important in ligand recognition and is directly involved in mediating cell-to-cell contact through Ca^{2+}-dependent interactions with cell-surface carbohydrates.[2]

Several ligands for E-selectin, which all contain sialyl Lewis^x-type glycans, have been identified including P-selectin glycoprotein ligand 1 (PSGL-1),[3] L-selectin,[4] CD66,[5] CD44,[6,7] and E-selectin ligand 1 (ESL-1).[8] However, E-selectin ligands on neutrophils have not been fully characterized to date. The best characterized selectin ligand is PSGL-1, which is found on leukocytes and platelets. PSGL-1 binds to P-, E-, and L-selectins in vitro and represents an important functional ligand for all of these molecules.[9]

Recent studies using double or triple selectin knockout mice revealed that selectins have both overlapping and distinct functions.[10] Single-selectin knockout mice showed only minor deficiencies in leukocyte recruitment in response to tumor necrosis factor α (TNF-α) or thioglycollate, suggesting distinct roles for the selectins in the inflammatory process. In contrast, double-mutant mice displayed more profound defects in neutrophil recruitment. For example, E- and P-selectin double-mutant mice showed an increased susceptibility to bacterial infection, with the majority of animals developing chronic inflammatory lesions of the oral mucosa and skin, suggesting that E- and P-selectin may function cooperatively.[11] The most severe deficiencies in neutrophil recruitment were found in E-, L-, and P-selectin triple-knockout mice, which had impaired neutrophil emigration, neutrophil rolling, and significant leukocytosis.[12]

Although the role of selectins in leukocyte recruitment is well established, it is now becoming clear that pathways engaged in response to E-selectin receptor engagement may trigger cell activation even though the molecular mechanisms remain to be defined. Engagement of selectin receptors has been reported to activate the mitogen-activated protein kinase (MAPK) pathway or activate cell-surface receptor-associated protein tyrosine kinases.[13]

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Recent findings from a number of studies suggest that soluble E-selectin may also engage selectin receptors. Soluble E-selectin levels are elevated in many chronic inflammatory conditions, including rheumatoid arthritis and asthma.[2,14] In addition, soluble forms of selectins are rapidly released from activated endothelial cells.[14] One possibility is that receptor shedding may represent a mechanism for limiting further inflammatory-cell recruitment by decreasing the availability of endothelial ligands for inflammatory cells. However, it is becoming clear that soluble E-selectin may activate inflammatory cells[15] and exert potentially proinflammatory effects.[16]

Early studies have suggested that selectin and platelet-activating factor (PAF) signaling act cooperatively to induce neutrophil adhesion to the endothelium.[17] E-selectin is known to reduce the rolling velocity of neutrophils in vitro.[18] In vivo work by Kanwar et al[19] found that low concentrations of exogenous PAF induced an increase in neutrophil adhesion in slow-rolling cells, whereas fast-rolling cells were unresponsive to the same concentration of PAF. Similarly, P-selectin has been shown to slow the rolling cells so that they are able to firmly adhere in the presence of lower concentrations of PAF.[20] These findings would suggest that reduced neutrophil rolling velocity following adhesion to selectins confers a higher ability to adhere in the presence of an appropriate stimulus. However, one possibility is that selectin receptor engagement may facilitate integrin-mediated "firm" adhesion following exposure to a second stimulus and that there could be communication between receptors for inflammatory mediators and those involved in adhesion at a molecular level.

We have previously demonstrated that Ca^{2+} mobilization induced by PAF in neutrophils, an early key event in the control of motility, respiratory burst, and degranulation, is prolonged in the presence of soluble E-selectin.[21] Neutrophil adhesion to β_2-integrin ligands (albumin-coated latex beads) induced by PAF but not by leukotriene B_4 (LTB_4) or formyl-Met-Leu-Phe (fMLP) was promoted by soluble E-selectin and this adhesion required PAF-induced Ca^{2+} mobilization from inositol 1,4,5-trisphosphate (IP_3)–sensitive intracellular stores. In this paper, we provide biochemical evidence for molecular cross-talk between these structurally distinct receptor pathways.

Materials and methods

Reagents

Hanks balanced salt solution (HBSS) was obtained from Life Technologies (Paisley, United Kingdom). Dextran T500 was obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). PAF, pertussis toxin, gadolinium(III) chloride, and wortmannin were obtained from Sigma-Aldrich (Poole, United Kingdom). ENA2 was purchased from Abcam (Cambridge, United Kingdom) and MRS1845 and ruthenium red from Tocris (Bristol, United Kingdom). U73122, U73343, LY294002 hydrochloride, LY303511, Fura2-AM, PP2, PP3, SB203580, SB202474, and PD98059 were purchased from Calbiochem (Nottingham, United Kingdom). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech.

Antibodies

Anti-TRPC6 was purchased from Alamone Labs (Jerusalem, Israel). Phospho-Src family (Tyr416) was purchased from Cell Signaling (Hertfordshire, United Kingdom) and anti–phopho-Akt1/PKBα (Ser473), clone 11E6, was obtained from Upstate Biotechnology (Milton Keynes, United Kingdom). Monoclonal anti–β-actin antibody was purchased from Sigma-Aldrich. Goat anti–mouse and –rabbit horseradish peroxidase (HRP)–conjugated antibodies were obtained from Dako (Ely, United Kingdom).

Expression and purification of E-selectin

Recombinant proteins of E-selectin were obtained using a baculovirus expression construct kindly provided by Dr Mike Bird (GlaxoSmithKline, Stevenage, United Kingdom). Recombinant human E-selectin, lacking the last 2 consensus repeats, was produced in a baculovirus insect-cell expression system as a C-terminal chimera with 2 protein A domains in tandem. High Five cells (BT1-TN-5B1-4 cell line, Invitrogen, Paisley, United Kingdom) were used to express recombinant E-selectin. High Five cells were cultured in Express Five serum-free media supplemented with L-glutamine and penicillin/streptomycin. High Five cells (9×10^6/75-cm^2 flask) were seeded into cell-culture flasks and left to adhere for 20 minutes. After attachment of the cells, the medium was removed and the cells were infected with recombinant virus at 2 PFU/cell. Three hours later, the medium was replaced with fresh medium. After 72 hours of incubation at 27°C, the culture supernatant was collected and stored at 4°C for further purification.

Recombinant proteins were then purified from High 5 insect-cell culture supernatants using IgG affinity column chromatography using the protein A domain in the recombinant protein. A column containing IgG-agarose was equilibrated with 5 column volumes of 0.1 M phosphate buffer, pH 8.0. The supernatant was applied to the affinity column, the column was washed with 2 column volumes of 0.1 M phosphate buffer, and eluted with 100 mM glycine in 500-μL fractions. Fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for protein content. The eluted proteins were then dialyzed against PBS (Ca^{2+} and Mg^{2+} free) overnight. A typical yield was 1.5 to 2.0 mg protein/250 mL supernatant.

Neutrophil isolation

Polymorphonuclear leukocytes were isolated from peripheral blood of healthy donors as described previously.[22] After centrifugation of citrated whole blood at 300*g* for 20 minutes and removal of platelet-rich plasma, leukocytes were separated from erythrocytes by dextran sedimentation using 0.6% (wt/vol) dextran T500. Polymorphonuclear leukocytes were then separated from mononuclear leukocytes using discontinuous isotonic Percoll gradients. Polymorphonuclear leukocytes were 95% to 98% neutrophils using morphologic criteria and cell viability was assessed by trypan blue exclusion.

Measurement of $[Ca^{2+}]_i$

Freshly isolated leukocytes were resuspended at 10^7/mL in HBSS (Ca^{2+} and Mg^{2+} free) and were incubated with 2 μM Fura2-AM at 37°C for 30 minutes in the dark. The cells were then washed twice to remove Fura2-AM and resuspended at 4×10^7/mL in HBSS (containing Ca^{2+} and Mg^{2+}). Intracellular calcium was monitored by Fura-2 emission fluorescence at 510 nm using 340/380-nm dual wavelength excitation in a Perkin Elmer (Beaconsfield, CA) luminescence spectrometer at 37°C with constant stirring. Calibration was performed after each experiment using 100 μL 0.1% (vol/vol) Triton X (R_{max}) and 10 mM EGTA (R_{min}). $[Ca^{2+}]_i$ was calculated from the 340:380-nm fluorescence ratio.[23]

RT-PCR analysis

Total RNA was extracted from freshly isolated neutrophils using the TRIzol reagent method per the manufacturer's instructions (Invitrogen, Life Technologies). Oligo(dT)–primed first-strand cDNA synthase was performed with Moloney murine leukemia virus reverse transcriptase (RT; SuperScript II, Promega, Madison, WI) using 500 ng mRNA as template in a total volume of 20 μL. The cDNA was then used for polymerase chain reaction (PCR) with *Taq* polymerase (Promega). Two pairs of specific primers each were used to detect the canonical transient receptor potential channel (TRPC) 1, 3, 4, and 6 and one primer pair was used for TRPC2 and 5; primers were synthesized by MWG Biotech (Ebersberg, Germany). The sequence of the primer pairs used along with the predicted size of their expected amplicons are as follows: TRPC1: forward 5′-ATGTATACAACCAGCTCTATCTTG-3′ and reverse 5′-AGTCTTTGGTGAGGGAATGATG-3′ (525 bp); TRPC1: forward 5′-TTCTGTGGATTATTATTGGGATGA-3′ and reverse 5′-CAGAACAAAGCAAAGCAGGTG-3′ (505

bp); TRPC2: forward 5′-TCTGGACCATGTTCGGTATG-3′ and reverse 5′-GCTACCTCGCTTTGCAGTC-3′ (565bp); TRPC3: forward 5′-CTGCAAATGAGAGCTTTGGC-3′ and reverse 5′-AACTTCCATTCTACATCACTGTC-3′ (388 bp); TRPC3: forward 5′-GCGAATTGTTAACTTTCCCAAATGC-3′ and reverse 5′-TCTTCCAAAAGTTCATAACGAAGGC-3′ (300 bp); TRPC4: forward 5′-ATTCATATACTGCCTTGTGTTG-3′ and reverse 5′-GGTCAGCAATCAGTTGGTAAG-3′ (329 bp); TRPC4: forward 5′-TTGCCTCTGAAAGACATAACATAAG-3′ and reverse 5′-CTACTAACACACATTGTTCACTGAG-3′ (300 bp); TRPC5: forward 5′-ACTTCTATTATGAAACCAGAGC-3′ and reverse 5′-GCATGATCGGCAATAAGCTG-3′ (289 bp); TRPC6: forward 5′-AAGACATCTTCAAGTTCATGGTC-3′ and reverse 5′-TCAGCGTCATCCTAATTTCCC-3′ (322 bp); TRPC6: forward 5′-ACAGATAATGCAAAACAGCTG-3′ and reverse 5′-ATGATGCTCTGGGCTTTG-3′ (244 bp); and GAPDH: forward 5′-TGCCTCCTGCACCACCAAGTG-3′ and reverse 5′-AATGCCAGCCCCAGCGTCAAAG-3′ (450 bp).

The reaction solution (50 μL) contained 0.4 μM of each primer (forward and reverse), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U *Taq* polymerase, and 1 μL cDNA. The PCR conditions were: 95°C for 10 minutes, followed by 35 cycles, each consisting of denaturation at 95°C for 1.5 minutes, annealing at 63°C for 2 minutes and extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. After PCR amplification, the reaction mixtures were applied to 1% (wt/vol) agarose gel for electrophoresis and DNA fragments were detected by ethidium bromide staining.

Western blot analysis

Neutrophils (5×10^{10} cells/L/sample) were lysed, following stimulation as detailed in the figure legends, in lysis buffer containing Tris HCl (100 mM, pH 8.0), NaCl (100 mM), EDTA (2 mM), Nonidet NP-40 (1% vol/vol), Na_3VO_4 (5 mM), NaF (50 mM), and protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes at 4°C. Samples were centrifuged for 20 minutes at 15 000*g* at 4°C and supernatants were reduced with electrophoresis sample buffer containing Tris HCl (0.25 M, pH 6.8), SDS (8% wt/vol), β-mercaptoethanol (10% wt/vol), glycerol (30% vol/vol), and bromophenol blue (0.02% wt/vol). Each sample was loaded onto a 10% SDS-polyacrylamide gel and proteins were separated and electrophoretically transferred to nitrocellulose. The membranes were then blocked for 1 hour in 5% (wt/vol) dried milk and probed with primary antibody overnight. After washing with Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST), blots were incubated with goat anti–mouse HRP (1:2500) or with goat anti–rabbit HRP (1:2500) antibodies for 1 hour at room temperature. The membranes were incubated with ECL reagent (Amersham Biosciences, Buckinghamshire, United Kingdom), placed under BioMax MS-1 x-ray–sensitive film, and processed through an x-ray developer (X-Ograph Imaging Systems, Wilts, United Kingdom).

Statistical analysis

Statistical analysis was carried out using the one-way ANOVA test with a Newman-Keuls multiple comparison posttest analysis, with statistical significance being achieved when P was below .05.

Results

Soluble E-selectin prolongs PAF-induced Ca^{2+} mobilization

Incubation of human neutrophils in the presence of soluble E-selectin did not induce Ca^{2+} mobilization (Figure 1A). However, in agreement with our previous studies,[21] preincubation of neutrophils with soluble E-selectin caused a subsequent sustained increase in $[Ca^{2+}]_i$ in response to PAF without affecting the initial increase of $[Ca^{2+}]_i$ (Figure 1A). To facilitate comparison of experimental data, we have calculated the area under the Ca^{2+} curve to provide a measure of the Ca^{2+} mobilization observed. Inhibition of soluble E-selectin interaction with neutrophils by ENA2, an anti–E-selectin monoclonal antibody that binds to the lectin domain of E-selectin, inhibited the sustained Ca^{2+} levels

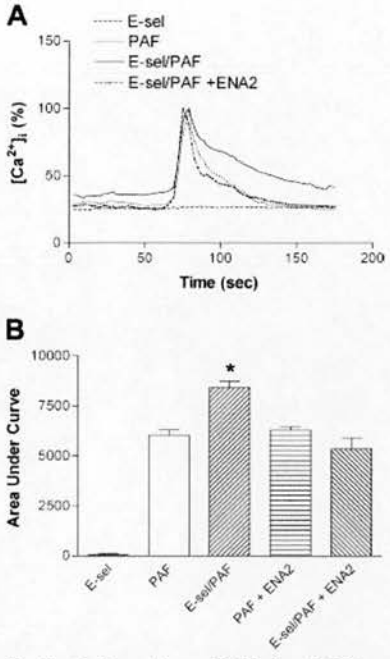

Figure 1. Soluble E-selectin prolongs PAF-induced Ca^{2+} mobilization. (A) Freshly isolated neutrophils were loaded with Fura2-AM (2 μM) for 30 minutes at 37°C in Ca^{2+}- and Mg^{2+}-free HBSS, then washed and resuspended in HBSS containing Ca^{2+} and Mg^{2+}, and then preincubated with or without soluble E-selectin (E-sel, 5 μg/mL) for 15 minutes at 37°C as indicated. For blockade of CD62E, ENA2 (1:50) and soluble E-selectin were preincubated for 1 hour before being added to neutrophils. Neutrophils were stimulated with PAF (100 nM) after recording "baseline" Ca^{2+} levels for 60 seconds. Data are shown as a representative trace of 5 separate experiments showing similar results and are expressed as percent of peak $[Ca^{2+}]_i$ following PAF stimulation (typically from 50 nM control levels to 2.5 μM following stimulation). (B) Traces from panel A have been integrated to calculate area under each curve using GraphPad Prism software (GraphPad, San Diego, CA) to compare the effects of various inhibitors. Data from 5 separate experiments that were performed and expressed as mean ± SEM. *Statistically different ($P < .05$) from PAF-treated controls.

without affecting the initial rapid rise in $[Ca^{2+}]_i$ observed in response to PAF treatment (Figure 1B). These data demonstrate that binding of soluble E-selectin via the lectin domain to a counterreceptor present on neutrophils is required for the prolongation of Ca^{2+} mobilization in neutrophils in response to PAF.

Using a range of preincubation times with soluble E-selectin, it was demonstrated that the effects of soluble E-selectin on prolongation of $[Ca^{2+}]_i$ in neutrophils were maximal by 15 minutes (Figure 2A), suggesting that downstream signaling pathways may be involved to induce prolonged Ca^{2+} mobilization in response to PAF rather than a rapid and direct physical interaction causing conformational changes. Furthermore, the effects of soluble E-selectin on prolongation of $[Ca^{2+}]_i$ levels were maintained whether soluble E-selectin was present or removed by washing; no significant difference was evident between samples (Figure 2B). Thus, the effects of soluble E-selectin are unlikely to be attributed to nonspecific effects such as sequestration/buffering of extracellular Ca^{2+} or changes in associated molecules that affect Ca^{2+} ion movements. These data suggest that soluble E-selectin triggers intracellular signaling pathways to modulate Ca^{2+} entry. We have previously shown that soluble E-selectin promotes Ca^{2+} mobilization and adhesion selectively to PAF but not to stimulation by fMLP or LTB_4.[21] Pretreatment of neutrophils with pertussis toxin, which ADP ribosylates G_i and G_0, was found to inhibit fMLP- and LTB_4-induced Ca^{2+} mobilization but had no effect on Ca^{2+}

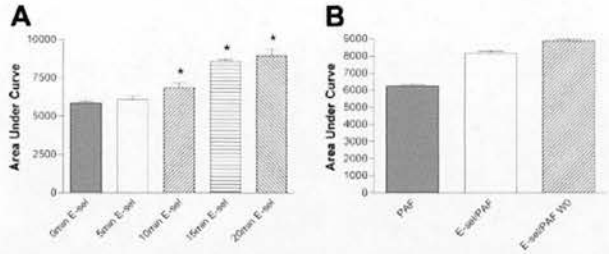

Figure 2. Effect of soluble E-selectin is time-dependent. (A) Freshly isolated neutrophils were loaded with Fura2-AM (2 μM) as for Figure 1 and preincubated with E-selectin (5 μg/mL) for various times as indicated. The cells were then stimulated with 100 nM PAF after 60 seconds of recording. Data from 3 separate experiments that were performed are expressed as mean area under the curve ± SEM. *Statistically different ($P < .05$) from PAF-treated controls. (B) Freshly isolated neutrophils were preincubated with soluble E-selectin and samples were either washed to remove E-selectin (WO) or used after 15 minutes of incubation. Data shown are expressed as mean area under the curve ± SEM from 3 independent experiments.

responses (Figure 3A), thereby suggesting a role for a pertussis toxin-insensitive G protein in mediating the effects of PAF.

E-selectin causes Ca^{2+} influx through a TRPC

PAF-induced Ca^{2+} mobilization is dependent on activation of phospholipase C and release of IP_3 because this response is sensitive to complete inhibition by U73122, a specific phospholipase C inhibitor (Figure 3B), whereas the inactive analog U73343 had no effect. Our previous studies also demonstrated that the initial PAF-induced $[Ca^{2+}]_i$ spike was abolished by TMB-8, which blocks Ca^{2+} release from intracellular stores, and that a relatively small second phase of Ca^{2+} mobilization was sensitive to inhibition by the receptor-operated channel inhibitor SKF96365.[21] Importantly, soluble E-selectin–induced promotion of Ca^{2+} mobilization in this latter phase was insensitive to SKF96365,[21] suggesting it occurs through distinct ion channels. We therefore sought to further define the molecular mechanism by which soluble E-selectin induced prolonged elevation of $[Ca^{2+}]_i$. Neutrophils treated with ruthenium red, an inhibitor of Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive stores, had no effect on mobilization of Ca^{2+} in response to PAF in the presence of E-selectin (Figure 3C). Chelation of extracellular Ca^{2+} by EGTA showed that only the E-selectin–mediated prolongation of $[Ca^{2+}]_i$ was sensitive to blockade and that PAF-induced Ca^{2+} release from stores was unaffected, indicating that soluble E-selectin affected Ca^{2+} influx rather than release from an intracellular store. The initial elevation of $[Ca^{2+}]_i$ in response to PAF, known to be due to release of Ca^{2+} from IP_3-sensitive stores, was unaffected by MRS1845, a store-operated channel inhibitor; however, soluble E-selectin–induced sustained $[Ca^{2+}]_i$ following stimulation with PAF was sensitive to inhibition by MRS1845 (Figure 3D). These results indicate that soluble E-selectin was allowing activation of a store-operated channel by PAF-induced Ca^{2+} store emptying, an effect we have termed "permissive" store-operated Ca^{2+} entry (SOCE). We next tested whether Gd^{3+}, a specific transient receptor potential family of cation channels (TRPC) inhibitor, would affect the E-selectin–induced $[Ca^{2+}]_i$ response to PAF. As shown in Figure 3C, the prolonged rise in $[Ca^{2+}]_i$ observed in the presence of soluble E-selectin was sensitive to Gd^{3+}, which is consistent with a role for TRPC in this response.

TRPC expression in polymorphonuclear leukocytes

To determine the profile of TRPC expression in human neutrophils, multiple specific primer pairs were used to screen for the presence of TRPC1-TRPC6 mRNA species in highly purified human neutrophils. The expression profile for members of the TRPC family is illustrated in a representative gel of RT-PCR products shown in Figure 4A. PCR products for TRPC6 were found in all neutrophil samples (n = 10), whereas TRPC3 was only found in 20% of samples. We did not observe signals for TRPC1, 2, 4, and 5 in any of the preparations, despite positive RT-PCR controls demonstrating that these PCR conditions were optimal. To confirm TRPC6 protein expression, we assayed crude membrane preparations from freshly isolated human neutrophils using Western blotting techniques. A specific antibody for TRPC6 revealed a strong band in the appropriate 90- to 100-kDa range, which could be blocked by a TRPC6-blocking peptide (Figure 4B), confirming the presence of protein and the RT-PCR data.

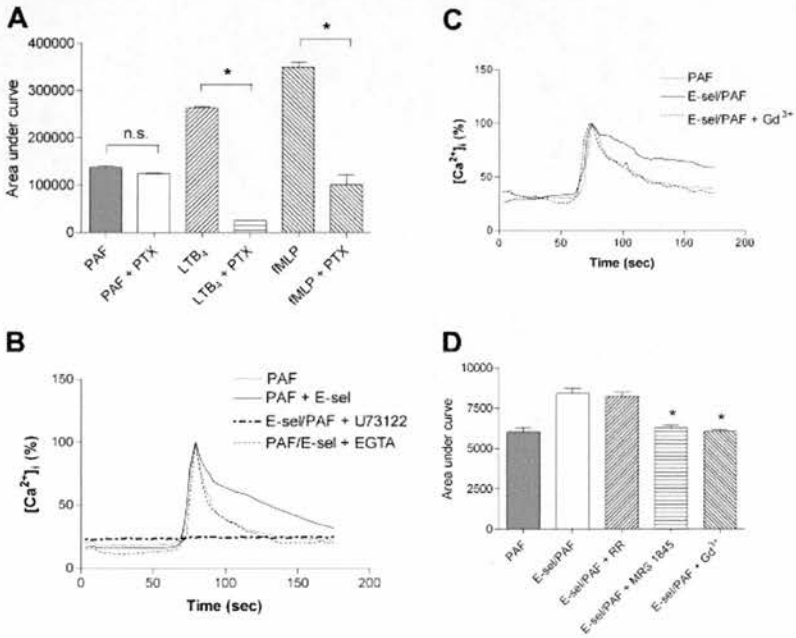

Figure 3. TRPCs mediate soluble E-selectin prolongation of PAF-induced Ca^{2+} mobilization. (A) Freshly isolated neutrophils were incubated with pertussis toxin (2 μg/mL) for 1 hour, washed, and then loaded with Fura2-AM (2 μM) for 30 minutes at 37°C prior to stimulation with 100 nM PAF. Data shown are expressed as mean area under each curve ± SEM from 3 separate experiments. *Statistically different ($P < .05$) from PAF/LTB_4- or fMLP-treated controls. n.s. indicates not significant. (B) U73122 (5 μM) and U73343 (5 μM) were added 5 minutes and EGTA (1.25 mM) was added 10 minutes before stimulation with 100 nM PAF. A representative Ca^{2+} trace from 3 separate experiments that were performed is shown. (C) Calcium traces showing the effect of calcium channel inhibitors on the prolonged $[Ca^{2+}]_i$ elevation induced by soluble E-selectin. Ruthenium red (RR; 20 nM), MRS1845 (2 μM) were added 5 minutes and $Ga^{3+}Cl_3$ (10 μM) was added 3 minutes before stimulation with 100 nM PAF. The calcium trace shown is representative of 3 separate experiments with similar results. (D) Bar graph representing area under the curves of the graph in panel C, calculated using GraphPad Prism software. Data shown are expressed as mean ± SEM from 3 separate experiments that were performed. *Statistically different ($P < .05$) from PAF/E-selectin–treated controls.

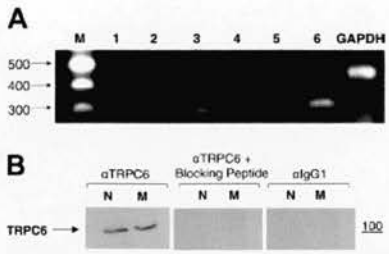

Figure 4. TRPC6 expression in human neutrophils. (A) Expression of TRP family members in isolated human neutrophils. Representative results of RT-PCR analysis with mRNA from a single isolation and cDNA preparation of neutrophils with each of the indicated primers are given and GAPDH was used as a positive control. Lanes are indicated as: M, markers; 1, TRPC1; 2, TRPC2; 3, TRPC3; 4, TRPC4; 5, TRPC5; and 6, TRPC6. (B) Western blotting of neutrophil (N) or mononuclear cell (M) membrane preparations stained with anti-TRPC6 antibody (1:400) revealed a strong band at the predicted molecular weight (100 kDa) as indicated. Specificity was demonstrated by incubation of the TRPC6 antibody with a 4-fold excess of the antigenic peptide exhibited no signal. Nonspecific binding was assessed using an IgG control stained with rabbit anti-IgG antibody (1:400). A representative immunoblot of 3 different experiments is shown.

E-selectin–induced SOCE is regulated by Src and PI-3K

It is now clear that critical regulatory elements that control TRPC activity include phosphorylation and Src family tyrosine kinases in particular.[24] We therefore used specific protein kinase inhibitors to test their involvement in soluble E-selectin–mediated SOCE. Inhibition of p38 MAPK by SB203580 (10 μM) or the negative control SB202474 (10 μM) had no effect on soluble E-selectin–induced Ca^{2+} influx following stimulation of neutrophils with PAF (Figure 5B). Similarly, a lack of effect by PD98059 (10 μM) on soluble E-selectin–induced modulation of Ca^{2+} influx identified that MEK1 was not involved in mediating these responses (Figure 5B). However, the specific Src family tyrosine kinase inhibitor PP2 (5 μM) selectively inhibited the soluble E-selectin–induced SOCE to levels observed in PAF-only stimulated neutrophils (Figure 5A-B), whereas PP3, the inactive analog, had no effect on soluble E-selectin–induced Ca^{2+} influx. These data suggest either a direct role for Src in modulating TRPC6 channel activity or potentially a role for Src in the downstream signaling events following soluble E-selectin binding to its putative receptor on neutrophils. Because PI 3-kinase is known to be a key regulator of ion channels in a variety of other cell types, we pretreated neutrophils with the specific PI 3-kinase inhibitors wortmannin (100 nM) or LY294002 (10 μM) and LY303511, an inactive structural analog. PI 3-kinase inhibition also inhibited the soluble E-selectin–induced SOCE to controls levels (Figure 5C-D), the inactive analog having no effect, thus identifying PI 3-kinase as a key regulator in the signaling pathway, which mediates the effects of soluble E-selectin on Ca^{2+} influx.

Western blot analysis of neutrophil protein lysates using a phosphorylation state-specific antibody (Tyr(P)^{416}), which correlates with Src activation, showed significant phosphorylation above control levels with soluble E-selectin treatment (Figure 6A). Interestingly, stimulation of neutrophils with PAF alone had no effect on the levels of phospho-Src. Pretreatment of neutrophils with PP2, prior to stimulation with soluble E-selectin, inhibited active phospho-Src to below control levels. In addition, LY294002 (10 μM) had no significant effect on soluble E-selectin–induced phosphorylation and activation of Src (Figure 6A), indicating that PI 3-kinase may be involved in a parallel pathway or acts downstream of Src in regulating Ca^{2+} influx. Soluble E-selectin caused activation of PI 3-kinase as assessed by a significant increase in phosphorylated Akt, a downstream target of PI 3-kinase, compared with control untreated cells, whereas PAF did not induce any Akt phosphorylation or activation (Figure 6B). Soluble E-selectin–induced increases in phospho-Akt levels could be inhibited completely by LY294002, confirming that it is a target of PI 3-kinase, and interestingly the Src tyrosine kinase inhibitor PP2 also showed complete inhibition of phospho-Akt accumulation following treatment with soluble E-selectin (Figure 6B). These data would support the hypothesis that the pathway that regulates permissive SOCE induced by soluble E-selectin is mediated primarily by Src with PI 3-kinase acting downstream.

Discussion

The selectin family of receptors is critical for the appropriate recruitment of neutrophils to sites of infection or tissue injury and

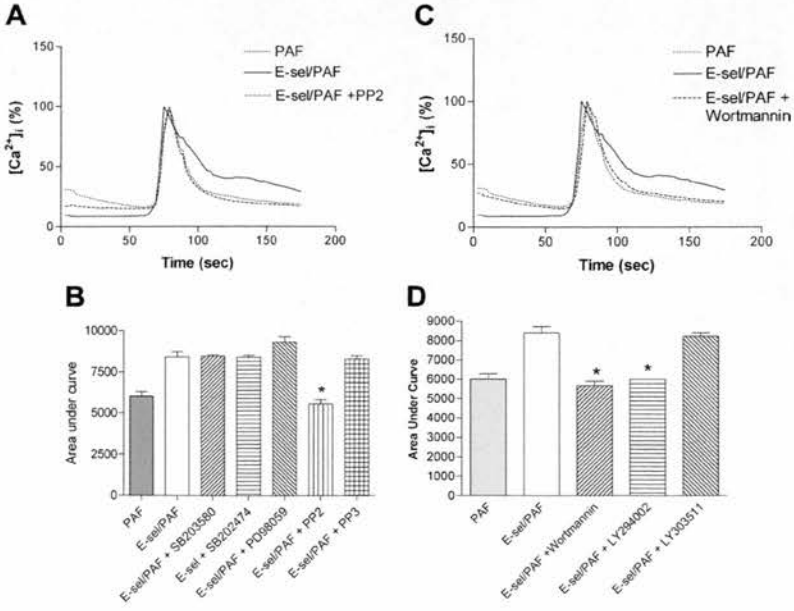

Figure 5. PI 3-kinase and Src kinase activity are required for soluble E-selectin Ca^{2+} mobilization. (A) PD98059 (10 μM), SB203580 (10 μM), SB202474 (10 μM), PP2 (5 μM), and PP3 (5 μM) were added concurrently with E-selectin for 15 minutes prior to stimulation with 100 nM PAF. The Ca^{2+} trace shown is representative of 3 separate experiments that were performed with similar results. (B) Bar graph representing area under the curves of graph in panel A, calculated using GraphPad Prism software. Data expressed as mean ± SEM of 3 independent experiments. *Statistically different ($P < .05$) from PAF/E-selectin–treated controls. (C) LY294002, LY303511 (10 μM, 5-minute preincubation), and wortmannin (100 nM, 15-minute preincubation) were added prior to stimulation with 100 nM PAF. The Ca^{2+} trace shown is representative of 3 independent experiments that were performed. (D) Bar graph representing area under the curves of graph in panel C, calculated using GraphPad Prism software. Data expressed as mean ± SEM from 3 separate experiments is shown. *Statistically different ($P < .05$) from PAF/E-selectin–treated controls.

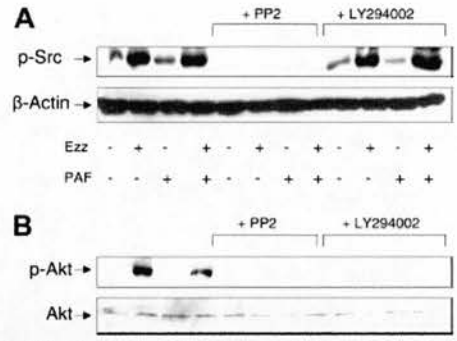

Figure 6. Soluble E-selectin induces Src and Akt activation in neutrophils. Freshly isolated neutrophils were incubated in the presence or absence of PP2 (5 μM, 15 minutes) or LY294002 (10 μM, 15 minutes), with soluble E-selectin (5 μg/mL, 15 minutes). Western blots of neutrophil lysates were carried out as described in "Materials and methods," and probed with (A) phospho-Src (Tyr 116) antibody (1:500) or (B) antiphospho-Akt1/PKBα antibody (Ser473; 1:200). To verify equal loading, the blots were probed with β-actin (1:10 000) or total Akt (1:2000). This figure is a representative blot from 3 independent experiments that were performed with similar results.

the initiation and progression of the inflammatory response. We have previously shown that soluble E-selectin acts to promote neutrophil adhesion, inhibit migration, and amplify destructive responses,[21] raising the possibility that elevated levels of soluble E-selectin in patients with inflammatory diseases, such as rheumatoid arthritis, and associated with tumor growth, have a proinflammatory effect.[25]

In leukocytes, PAF acts through a specific G protein-coupled receptor to induce chemotaxis. Neutrophil activation by PAF has been shown to be insensitive to pertussis toxin, implicating a $G_{q/11}$ family member. Neutrophil responses to stimulation by LTB_4 and fMLP are sensitive to pertussis toxin, suggesting G_o or G_i involvement.[26-28] β_2-Integrin-mediated neutrophil adhesion to albumin-coated latex beads induced by PAF but not fMLP and LTB_4 was promoted by soluble E-selectin.[21] Furthermore, we have also shown that soluble E-selectin specifically prolongs elevation of $[Ca^{2+}]_i$ in response to PAF but not fMLP or LTB_4.[21] We have demonstrated that pertussis toxin does not affect PAF-induced Ca^{2+} mobilization but abolishes fMLP- and LTB_4-induced Ca^{2+} mobilization. Taken together, this would indicate that only signals from a pertussis toxin-insensitive $G_{q/11}$-coupled receptor such as the PAF receptor are able to communicate through a G protein-derived signal to allow prolonged Ca^{2+} signaling to occur in the presence of soluble E-selectin.

We provide important new information relating to the mechanism by which soluble E-selectin prolongs PAF-induced Ca^{2+} signaling in neutrophils. Stimulation of neutrophils with PAF causes primarily a rapid release of Ca^{2+} from IP_3-sensitive stores and a relatively minor influx of Ca^{2+} through SKF96365-sensitive channels, most likely receptor-operated channels.[21] Soluble E-selectin alone does not cause release of Ca^{2+} from intracellular stores or via Ca^{2+} influx but acts to prolong Ca^{2+} signals induced by PAF receptor ligation in a novel manner by allowing "permissive" SOCE. We have demonstrated that prolongation of PAF-induced Ca^{2+} signaling by E-selectin is due to Ca^{2+} influx due to its sensitivity to blockade by EGTA rather than further release from ryanodine-sensitive intracellular stores. Furthermore, an obligate requirement for IP_3-mediated release of Ca^{2+} from intracellular stores to act as a trigger for the Ca^{2+} influx permitted by soluble E-selectin was demonstrated by inhibition of phospholipase C causing a complete loss of any Ca^{2+} signaling. In addition, the susceptibility of E-selectin–permitted Ca^{2+} influx to blockade by MRS1845, a store-operated channel (SOC) inhibitor, identified this as SOCE. A role for TRPCs as candidates for mediating this novel SOCE was proposed based on the ability of Gd^{3+} to cause selective inhibition of prolonged Ca^{2+} entry following PAF stimulation in the presence of soluble E-selectin. Proteins homologous to the *Drosophila* transient receptor potential gene *(trp)* Ca^{2+} channels that assemble into tetrameric ion channels are known to be involved in the generation of store-operated Ca^{2+} entry (SOCE). Our RT-PCR studies found that only TRPC3 and TRPC6 mRNA were expressed in polymorphonuclear leukocytes (Figure 4A), in agreement with Heiner et al.[29] TRPC6 appears to represent the principal TRPC family member present, being detected at both the level of mRNA and protein.

Thus, soluble E-selectin acts to promote a novel form of molecular cross-talk involving TRPCs that allow a putative E-selectin receptor to influence PAF-induced signaling pathways. Our data also suggest that both $G_{q/11}$- and soluble E-selectin–mediated signals are required to communicate with TRPC6 before release of Ca^{2+} from intracellular stores can trigger SOCE. We are currently investigating potential mechanisms for this effect, for example, whether a regulatory protein becomes recruited to TRPCs to permit SOCE, or promotion of a physical interaction between IP_3 channels in the endoplasmic reticulum (ER) with TRPC in the plasma membrane, or alternatively, TRPCs may become sensitized to the signals that mediate SOCE, such as calcium influx factor (CIF).[30] It has recently been shown that TRPC6 is externalized to the plasma membrane by the stimulation of a G_q protein-coupled receptor,[31] and it has also been shown that expression of TRPC6 in COS cells increases Ca^{2+} entry in response to stimulation of a G_q protein-coupled receptor.[32] We are therefore currently investigating whether soluble E-selectin preincubation leads to the up-regulation of TRPC6 on the cell surface and if this is sensitive to activation via G_q-coupled receptor-induced signals specifically.

The recent finding that diacylglycerol directly activates TRPC3 and TRPC6 may represent an alternative mechanism for activation of these channels via phospholipase C-linked receptors,[33] allowing regulation to occur through a lipid mediator. Recent studies have shown that tyrosine phosphorylation by Src family protein tyrosine kinases represents a potential regulatory mechanism of TRPC6 activity.[24] It has been suggested that 2 simultaneous events, opening of the channel by DAG and modulation by Src-induced tyrosine phosphorylation, contribute to the efficient influx of calcium through TRPC6. We found that PP2 specifically inhibited soluble E-selectin–mediated SOCE without affecting PAF-induced responses. In parallel, soluble E-selectin caused phosphorylation and activation of Src, which was sensitive to inhibition by PP2 but was unaffected by PI 3-kinase inhibition. These findings strongly suggest that Src activity is involved in modulating TRPC6 activity to regulate Ca^{2+} influx in human neutrophils.

Inhibition of PI 3-kinase selectively blocked the soluble E-selectin–induced SOCE in neutrophils. Several potential intracellular regulatory motifs have been identified on TRPC6 including PI3K-SH2 recognition domains, suggesting a mechanism by which these channels might interact with the PI 3-kinase signaling pathway.[34] Several groups[35,36] have discovered that the PI 3-kinase lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP_3) mediates calcium influx through a mechanism independent of phospholipase C (PLC) activity or store depletion in several cell lines. The activation of receptor tyrosine kinase cascades leads to the membrane colocalization of PLCγ and PI 3-kinase, both of which use phosphatidylinositol 4,5-bisphosphate (PIP_2) as a substrate to generate IP_3 and PIP_3, respectively. These 2 signaling intermediates

trigger the activation of calcium channels at different cellular compartments, giving rise to elevated levels of $[Ca^{2+}]_i$. Soluble E-selectin was demonstrated to cause an increase in phospho-Akt, a downstream target of PI 3-kinase. We would speculate that PI 3-kinase acts to modulate TRPC6 activity and that PI 3-kinase lies downstream of Src in the regulation of soluble E-selectin–mediated permissive SOCE.

Several putative glycoprotein selectin ligands have been isolated from hematopoietic cells using in vitro affinity purification techniques, but the exact identity and contribution of physiologic E-selectin ligands on neutrophils is unknown.[37] In this paper, we have demonstrated that E-selectin binds via the lectin domain to cause permissive SOCE in neutrophils in response to PAF, presumably through a putative E-selectin receptor present on neutrophils. A cell-adhesion molecule suggested to play a role in E-selectin adhesion is CD66 or carcinoembryonic antigen (CEA). Neutrophils are known to express several CEA family members, which are all highly glycosylated molecules with multiple sialyl and fucosyl residues. In preliminary experiments, CD66 ligation with antibodies caused prolonged PAF-induced Ca^{2+} mobilization in a similar manner to that caused by soluble E-selectin (data not shown). We are currently investigating the possibility that CD66 and other adhesion receptors need to coengage via soluble E-selectin to regulate Ca^{2+} signaling in response to PAF.

Receptor-mediated activation of leukocytes by inflammatory stimuli requires Ca^{2+} mobilization and influx as a critical common activation mechanism. Selective modulation of distinct components of these Ca^{2+} signals may represent potentially attractive strategies for developing anti-inflammatory drugs to attenuate leukocyte activation. Our report is the first demonstration of soluble E-selectin causing permissive SOCE to occur following activation of neutrophils by PAF and that this SOCE most likely occurs through TRPC6. We have identified a novel form of permissive SOCE induced by soluble E-selectin in human neutrophils, which occurs through a Src/PI 3-kinase–dependent pathway and also requires a $G_{q/11}$-derived signal to sensitize or prime TRPCs to open on increased intracellular Ca^{2+} and depletion of Ca^{2+} stores, but the precise order of these molecular events is yet to be fully explored (Figure 7). This novel mechanism of molecular cross-talk integrates signals from pertussis toxin-insensitive $G_{q/11}$-coupled receptors and TRPCs and could be critical for fine tuning adhesion and migratory responses during neutrophil recruitment during inflammation.

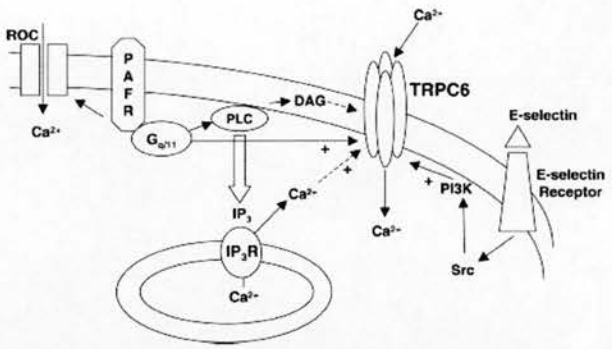

Figure 7. Schematic model of intracellular communication between E-selectin receptors and PAF receptor. PAF binds to its $G_{q/11}$ protein-coupled receptor resulting in activation of PLC, leading to cleavage of PIP_2 and generation of membrane-retained DAG and cytosolic IP_3. DAG can directly activate TRPC6.[34] Soluble $InsP_3$ activates the IP_3R on the endoplasmic reticulum to release intracellular Ca^{2+}. These responses result in the initial rapid increase of $[Ca^{2+}]_i$. E-selectin interacts with E-selectin receptors on the neutrophil surface, permitting PAF-induced Ca^{2+} mobilization to communicate with TRPC6 and allowing permissive SOCE to occur. Modulation of this Ca^{2+} channel involves Src and PI 3-kinase pathways. ROC indicates receptor-operated channel; G, G protein-coupled receptor.

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